EDITOR'S VIEWPOINT

The behavior of materials used in dental laboratories is complicated because many of their reactions are not simple chemical phenomena. Hygroscopic expansion of dental investments, one of these phenomena, has been studied by various methods. Ryge and Fairhurst (p. 499) used x-ray diffraction studies on these interesting materials and, producing hygroscopic expansion by water and other liquids, demonstrated that it is not caused by hydration. They explain the "hygroscopic" expansion on the basis of physical properties of the materials, fluid adsorbed between inert gypsum crystals thickening their surface films and forcing the crystals apart.

The effect of various types of dental instruments on the smoothness of dental instruments of cavity walls has been expressed from time to time but few studies have been reported. Peyton and Mortell (p. 509) used a shadowing technic to demonstrate the roughness of cavity walls after use of various rotating and hand instruments. The roughness of the walls is apparent from the photographs, but whether greater smoothness is desirable has not been demonstrated. The relative retention and the clinical desirability of different types of cavity wall finish challenge the investigator in the field of physical properties.

Crawford and Larson (p. 518) subjected extracted teeth with metal restorations to fluids containing radioactive calcium and then studied its penetration in radiographs. The penetration of the fluids around serviceable restorations is apparent. The authors ask why these restorations are serviceable and what prevents recurrent decay in the regions of fluid penetration.

Lefkowitz and Swayne (p. 523) continued their in vitro cultivation of tooth germs by studying their growth "on the clot" as opposed to previous "in the clot" explants. This study throws some doubt on the generally accepted concept that odontoblasts and ameloblasts exert organizing influences on each other. In tissue culture this observation was not confirmed. Another interesting finding was the appearance of monocyte-like cells in the cultures at the time liquefaction began. One must realize that these cultures have no blood supplies to understand the importance of such cells which possibly are "reticuloendothelial" in nature.

The development of the squamoso-mandibular articulation of the rat (Cunat, Bhaskar and Weinmann [p. 533]) is a detailed description of the embryology of this structure as observed in normal and abnormal animals from 16 days insemination age until 30 days after birth. The details will be of value to anatomists and to all investigators and clinicians whose problems are concerned with the "mandibular joint."

Bixler, Webster and Muhler (p. 547), by a study of the adrenals after removal of the major salivary glands of weanling rats, have demonstrated an increase in the adrenocortical activity, suggesting a moderate ACTH stimulation, and a retardation of the development of the female reproductive system. If the salivary glands show this hormone-like activity in rats, their function in man should be reviewed and re-evaluated.

The sequence of calcification of the mandibular molars and premolars of a group of children seen periodically in a longitudinal study was observed by radiographs. Garn, Lewis and Shoemaker (p. 555) found that of the 5 teeth investigated the first molar was invariably first to begin calcification, the first premolar second and the third molar last. The time of initial calcification in the second premolar and second molar had different tendencies in boys than in girls, and boys showed more predominance of approximation of initial calcification of these 2 teeth. Studies on siblings suggest a genetic influence on which of the two teeth begin to calcify first and a gradual evolutional change from M_2P_2 sequence to P_2M_2 sequence is proposed. This is the type of general study to which the interested and capable dentist may contrib-

ute and from which he can derive useful dental information.

Oršós and Bartha (p. 562) observed that when sleep was induced in albino rats the rate of eruption of the incisors was increased. To test whether or not the lack of intrusive forces during sleep, i.e., the failure to use or bite upon the teeth, was the cause of this accelerated eruption, the teeth on one side were ground out of occlusion each week. The authors conclude that increased eruption rate in animals in which prolonged sleep has been induced probably is not due to reduced intrusive forces. They have not found the cause of this eruption rate change.

Gans, Engel and Joseph (p. 566), by an electrometric method, demonstrated that the density of the negatively charged colloid is decreased in early months of pregnancy and that the tissues return to normal after parturition. The changes, present in all patients, may require a triggering mechanism such as local trauma or general reduction in resistance to result in clinical manifestations. That the gingival tissues do respond to general influences is well demonstrated in the familiar dilantin reaction and, although some authors have questioned the specificity of the hormonal influence, it appears to be well established.

Green and Dodd (p. 572), from a study of caries-susceptible and truly caries-immune individuals, appear to have demonstrated that the saliva of immunes suppresses the number of lactobacilli present in saliva and almost eliminates those with colony morphology other than smooth. The mechanism appears to be a selection. While the authors propose that the mechanism may lower the total cariogenic potential of the oral flora, they point out that it does not imply that lactobacilli are the sole organism that may be active in the process of caries. Other organisms, however, were not significantly reduced in numbers in the saliva of caries-immune subjects.

Rosen, Hunt and Hoppert (p. 586) found lactobacilli more frequently in the mouths of caries-susceptible rats than in caries-resistant rats from the Michigan State "caries-breeding" colonies. The occurrence of lactobacilli in young caries-resistant rats, although always lower than in caries-susceptible groups, shows a marked drop after 50 days of age. There is also a statistically significant lower incidence of oral lactobacilli in resistant animals developing no caries as compared with resistants that developed caries. The authors suggest that lactobacilli may cause caries in susceptibles but are not important in the carious process in resistant rats. Different organisms may be important in resistant animals. It has also been shown (Green and Dodd, p. 572) that the cariogenic flora may differ in susceptible and immune human subjects.

Hargreaves and Manly (p. 591) have evaluated, with a large group of agents, their "salivary sediment technic" for measuring acid inhibition. The results have been compared with the saliva-glucose mixture method. Higher concentration of inhibiting agents are required in the salivary sediment method than are necessary to show inhibition of acid formation with the saliva-glucose mixture. Both methods should be recognized as screening technics for acid formation inhibiting effects, not as caries inhibition tests.

Onisi and Kondo (p. 596) placed acrylic plates resembling horseshoe dentures without teeth, in the palates of individuals who wore them 4 days. Daily salivary lactobacillus counts made for six days before the experiment were used as base lines. Counts made during the period of wearing the plates were significantly increased and dropped again after the plate was removed. The findings suggest the importance of environment in determining individual lactobacillus counts and may explain why counts may be increased in individuals with inactive, but unrestored, carious lesions, and in patients with partial dentures, or orthodontic appliances.

Schatz, Martin and Karlson (p. 603) consider the oral flora as unique with every changing environmental condition. They investigated the cultural characteristics of organisms selected from the oral microflora. Using the Warburg technic they demonstrated the potential of this approach which has been utilized to study bacteriologic aspects of other mixed populations.

Rae and Clegg (p. 612) were unable to correlate salivary lactic acid production with salivary lactobacilli counts. They suggest that lactic acid production is a poor criterion for lactobacillus concentration.

Muhler and Bixler (p. 615) found that desalivated rats fed a cariogenic corn diet

did not develop dental caries when their maxillary molars had been removed, although those with their maxillary molars present did. They express the belief that a factor other than the absence of saliva is necessary to initiate caries in the rat, but the lack of saliva will increase the rate of caries activity. The "food impaction" or "fracture" property of certain diets that initiate caries in the rat has long been recognized, but some question has been raised as to its authenticity. This work tends to reaffirm the coarse corn diet concept and to assign different roles to the physical properties of the diet and the saliva.

Although real differences in caries susceptibility exist in the Hunt-Hoppert series of albino rats, the search for basic factors has proved elusive. It had been noted, in passing, that the susceptibles had wider fissures in their molars than the resistants. A study of this factor by Kifer, Hunt, Hoppert and Witkop (p. 620) indicates that the difference is significant. It is postulated that these wider fissures facilitate food impaction while, in human beings (Bossert, W. A.: *J. D. Res.* **16**: 63, 1937) quite the opposite condition appears to prevail. While this does not invalidate work on rat caries it does highlight the danger of making transfers of information from experimental animals to human subjects too hastily.

Weinreb and Ben-Shushan (p. 630) report the caries experience of 7,727 young Israeli males. The number of D.M.F. teeth appear low in comparison with reported D.M.F. from other countries.

Potgieter, Morse, Erlenbach and Dall (p. 638) studied the relationship between D.M.F. of school children and food intake as recorded by the children over a 7-day period. As may be expected, when the relatively gross technic of D.M.F. by teeth is used on 864 children and the dietary habits are obtained from records kept by the children, precise conclusions are unwarranted. The authors carefully avoid any effort to draw conclusions but point out that the D.M.F. rate is lower among the children recording more complete food intake (as judged by serving of the "basic seven-group items"), that the D.M.F. goes down as milk intake increases to 5 cups per day but increases above this level and that small but inconclusive relationships were recorded between candy and between-meal snacks and D.M.F.

An interesting group, from the viewpoint of oral hygiene methods, was studied by Sanjana, Mehta, Doctor and Baretto (p. 645). Of 1,445 individuals of Bombay 83 per cent had generalized periodontal disease. One-half used tobacco on the finger for their oral hygiene, others using the datan (a fresh chewed twig), toothbrushes and paste, the fingers with powder, the fingers with charcoal, or the toothbrush with powder. The method of oral hygiene did not seem to affect the final outcome, significantly, as practically all of the subjects over 30 years of age had generalized periodontal disease. This study indicates that in Bombay more than simple oral hygiene by the individual is necessary to prevent periodontal disease.

Laboratory evidence of systemic derangement, generally, has not been demonstrable in cases diagnosed as periodontosis. Karshan, Tenenbaum and Friedland (p. 648) have demonstrated consistently low urinary estrogen values in the urine, obtained on the day of presumed evaluation from 7 women with severe periodontosis. The urinary estrogen output was lower in these patients than in 3 women with mild and 8 with moderate periodontosis. This limited evidence is suggestive, and further studies of this factor appear to be justified in young women with periodontosis.

Mitchell and Johnson (p. 651) appear to have produced "gingival plaque" at will in hamsters. A high carbohydrate diet given for three months resulted in plaque material at the gingival margin of upper molars while feeding noncariogenic laboratory chow for 2 weeks led to its disappearance. The plaque re-formed after two weeks on the high carbohydrate feed. This plaque, consisting largely of aciduric organisms, is suggested as a screening device for possible anti-caries agents.

Fajarda, Grossman and McShane (p. 656) report on the results of tests with several relatively less known antibiotics and antiseptics against 4 microorganisms frequently found in root canal therapy. The value of combining agents is suggested. The method of their use is not discussed.

H. B. G. R.

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH
1957 MEETING

The next general meeting will convene at the Claridge Hotel, Atlantic City, N. J., on Thursday, Friday, and Saturday, March 21, 22, and 23, 1957. The Dental Materials Group of the Association will meet on Friday, Saturday, and Sunday, March 22 to 24. The American Association of Dental Schools will meet at the same place beginning on Sunday, March 24.

You are reminded that the official headquarters of the Association are located at 129 East Broadway, Louisville 2, Ky., and all mail except that directed to specific officers and committees should go to this address care of Dr. Dan Y. Burrill, Assistant Secretary-Treasurer.

The Novice Award will be offered again under approximately the same conditions as last year.

Nominations to membership in this Association must be in the hands of the Secretary or the Nominating Committee by February 15, 1957, if they are to be considered at the March, 1957, meeting. *Completed* nomination forms with supporting material should be sent to the Assistant Secretary-Treasurer, Dan Y. Burrill, 129 E. Broadway, Louisville 2, Ky., or to the Chairman of the Membership Committee, Dr. R. S. Manly, 136 Harrison Ave., Boston 15, Mass.

Abstracts of the program items of the last meeting will not be printed in the JOURNAL OF DENTAL RESEARCH, as has been the previous practice. However, copies of the preprinted abstracts are available as long as they last. Please request copies from Dr. Burrill, Louisville. Price, postpaid, $2.00.

Address all communications with reference to the coming meeting to:

Dr. Dan Y. Burrill, Assistant Secretary-Treasurer,
129 East Broadway,
Louisville 2, Ky.

HYGROSCOPIC EXPANSION

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INTRODUCTION

THE phenomenon of hygroscopic expansion was called to the attention of the dental profession by Scheu[1] in 1932. Since this time numerous investigators[2-8] have published data illustrating the manipulative factors determining the amount of hygroscopic expansion. Degni[6] explained the phenomenon observed on the basis of the colloidal theory for the setting of plaster of Paris. Landgren and Peyton[7] gave an excellent account of several factors affecting the hygroscopic expansion but did not discuss the mechanism thereof. Lyon, Dickson, and Schoonover[8] illustrated the mechanism of hygroscopic expansion from several new aspects and concluded:

> On the basis of the data presented it is believed that hygroscopic expansion of dental investment is a continuation of the normal setting expansion during the period when the investment is in a semisolid state. This continued expansion is produced by the introduction of additional water which permits further hydration of calcium sulphate at this time. The inert filler present in the investment weakens its structure and thus permits the precipitating hydrate crystals to expand the mass of material.

It is the purpose of this report to present the results of x-ray diffraction data illustrating the rate of hydration of different plaster products under hygroscopic and nonhygroscopic conditions, and to present other observations on the phenomenon of hygroscopic expansion.

RATE OF HYDRATION

Diffraction data were obtained from 19 commercially available dental casting investments, dental stones, and plasters in their hemihydrate form and in their dihydrate form after normal setting. The position and relative intensities of the diffraction lines characteristic of the hemihydrate and dihydrate were verified by comparison with those of pure samples of calcium sulfate hemihydrate and dihydrate produced by precipitation.[9, 10]

The casting investments, stones, and plasters were then mixed with water and permitted to set under different hygroscopic conditions, and x-ray diffraction data were again obtained. Details of the experimental conditions were as follows:

Diffraction data were obtained using a General Electric XRD-3 recording x-ray diffractometer operated with a copper tube at 50 kv., 10 Ma. A beam slit of 3 degrees

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was used, with a medium resolution soller slit, 0.2 degree detector slit, and 0.0007 inch Nickel filter. The spectrogoniometer was rotating at 2 degrees per minute and the chart was moving at 24 inches per hour. Recordings covered three decades, with a time constant of 6 seconds for the first decade, 3 seconds for the second decade, and 0.4 second for the third decade. Specimens were prepared from plaster, stone, and investment mixed under standardized conditions of proportioning and spatulation.

An aluminum frame placed upon a microscope slide was used as a sample holder (Fig. 1). Mixed plaster, stone, or investment was poured in the aluminum frame; then the excess was scraped off and another microscope slide was placed on top of the specimen to serve as a backing for the sample. When a liner was used, a sheet of wet or dry asbestos was placed on top of the specimen before the top (back) microscope slide was placed. In all instances the front (lower) microscope slide was removed as soon as the specimen was rigid enough to permit its removal (about 9 minutes after the start of the mix).

Recordings were started 10 or 15 minutes after start of mix. In order to obtain earlier readings this technic was modified slightly. A sheet of 0.00025 inch Mylar (Dupont) was attached to the front (lower) surface of the aluminum frame with No. 666 double-coated Scotch tape, thus forming a window and front wall which would support the specimen while soft (before initial set) so that the specimen could be placed vertically on edge in the sample holder of the x-ray diffractometer as early as 1½ minutes after the start of the mix. The Mylar window technic caused a slight shift in background but did not change the relative intensities of the recorded lines. The early recordings did not furnish additional information with respect to the phenomenon of hygroscopic or nonhygroscopic setting and, consequently, most data reported here are based on recordings started at 10 minutes, obtained with the use of the Mylar window. However, the early recordings aided in determining that the changes reported here cover the significant part of the setting period.

The data used for the curves in Fig. 2 were obtained by repeated scanning from $2\theta = 10$ degrees through $2\theta = 16$ degrees. In addition, other intervals were scanned, and several of the significant hemi- and dihydrate lines were also recorded by continuous scanning back and forth (using the increase-decrease switch of the spectrogoniometer) over a few degrees 2θ interval during the whole setting period.

It was found that the rate of formation of dihydrate and the rate of disappearance of the hemihydrate would vary with spatulation and with water/powder ratio. Under any set of experimental conditions, the final intensity of the dihydrate lines would reach higher values when the setting took place under normal conditions than when the setting took place under hygroscopic conditions.

It was found that the rate of change of different x-ray diffraction lines of each compound (hemihydrate or dihydrate) would vary somewhat, the reflections from planes of low Miller indices (high d-values) in general being most sensitive to the effect of hygroscopic conditions.

HYGROSCOPIC EXPANSION WITHOUT WATER

X-ray diffraction data and dimensional change determinations were made using the "water-added technique" described by Mahler.[11] "Hygroscopic expansion" was observed after addition of 2.5 ml. of ethyl alcohol, glycerine, transformer oil, and light machine oil.

The experimental procedure employed for determining dimensional changes upon setting was the following:

Weighed amounts of investment and distilled water were spatulated by hand. Of the mixed investment, 70 Gm. was placed in a cylindrical Korogel mold approximately the

Fig. 1.—Aluminum frame sample holder on microscope slide.

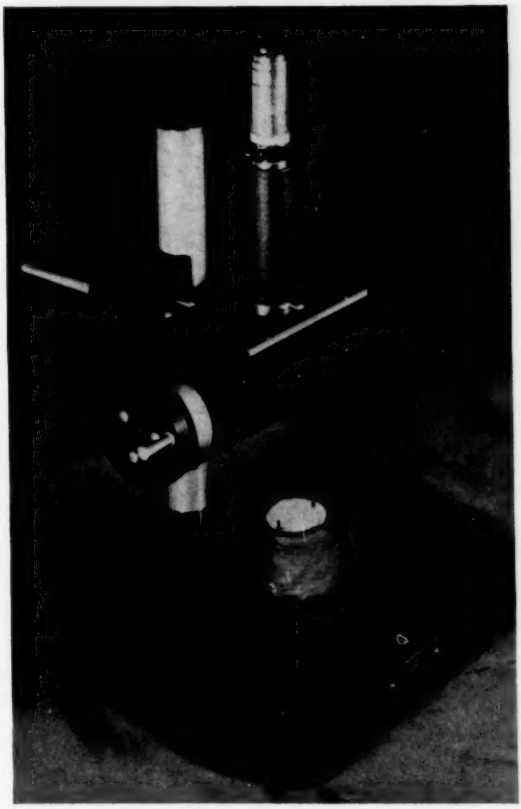

Fig. 2.—Gaertner micrometer microscope with Korogel mold filled with investment, with brass reference pins in position.

size of an inlay casting ring. Brass reference pins (14 gauge, 15 mm.) with a fine cross inscribed on the highly polished end surface were inserted into the investment approximately 32 mm. apart. This technic was later modified using commercially available rubber cylinders furnished by The Ransom & Randolph Company. The amount of investment used was 45 Gm. Reference pins were inserted 23 mm. apart. The mold was oriented on the stage of a Gaertner micrometer microscope such that the reference pins were in focus and in alignment with the microscope (Fig. 2). The method was later modified such that photographic records were obtained by a 35 mm. camera. Readings of the films were carried out on the micrometer microscope.

To determine whether water added to the specimens during the initial setting period takes part in the hydration process, 70 Gm. specimens were made as previously described. Different quantities of water (1 ml., 2.5 ml., and 3.5 ml.) were added to the specimens during the initial setting period, while others were allowed to set without the addition of water. The weight was again determined after 48 hours of storage in air.

RESULTS

Fig. 3 shows hydration curves for Kerr's Cristobalite Investment (K-curves, Graphs *A* and *C*) and Ransom & Randolph Hygroscopic Investment No. 2 (R-curves, Graphs *B* and *D*). Graphs *A* and *B* show absolute intensity plotted as a function of time for the 14.7 degree hemihydrate lines (designated by H) and the 11.6 degree dihydrate lines (designated by D). In Graphs *C* and *D*, relative intensity, I_H/I_D, is plotted as a function of time for the same data. The experimental conditions are listed in Table I.

TABLE I

KERR'S CRISTOBALITE				R & R HYGROSCOPIC NO. 2			
W/P RATIO	SPATULATION TIME	SETTING CONDITIONS	DESIGNATION	W/P RATIO	SPATULATION TIME	SETTING CONDITIONS	DESIGNATION
.35	2 min.	Normal	K1	.26	2 min.	Normal	R1
.38	2 min.	Normal	K2	.30	2 min.	Normal	R2
.38	2 min.	Hygroscopic	K3	.30	2 min.	Hygroscopic	R3
.38	30 sec.	Normal	K4	.30	30 sec.	Normal	R4

Fig. 4 shows typical expansion curves for R & R hygroscopic casting investment for normal setting, and for setting after additions of 1 ml. and 2.5 ml. of water, 10¼ minutes after start of mixing. The values obtained after 1 hour were: normal setting expansion, 0.44 per cent; 1 ml. of water added, 2.0 per cent; and 2.5 ml. of water added, 3.08 per cent.

Fig. 5 shows typical expansion curves for R & R hygroscopic casting investment, as effected by addition of 2.5 ml. of liquid at 10¼ minutes after start of mixing. The values obtained after 1 hour were: normal setting expansion, 0.44 per cent; 3-in-1 oil added, 0.95 per cent; glycerine added, 1.23 per cent; 100 per cent ethyl alcohol added, 2.29 per cent; and distilled water added, 3.08 per cent.

The data chosen for illustration in Figs. 4 and 5 were as close as possible to the average values for 6 individual determinations (for example, 6 experiments identical with B [Fig. 4] gave 1-hour expansion values ranging from 1.94 per cent to 2.04 per cent).

Kerr Cristobalite, water/powder ratio: 0.38, spatulated for 1 minute, initial reading at 11½ minutes; 1 ml. liquid added at 12 minutes, gave the

following expansion values: distilled water added, 2.1 per cent; 100 per cent ethyl alcohol added, 1.4 per cent; glycerine added, 0.8 per cent; light transformer oil added, 0.75 per cent; 3-in-1 oil added, 0.7 per cent; and normal setting expansion was recorded as 0.4 per cent. All values were reproducible to ± 0.05 per cent.

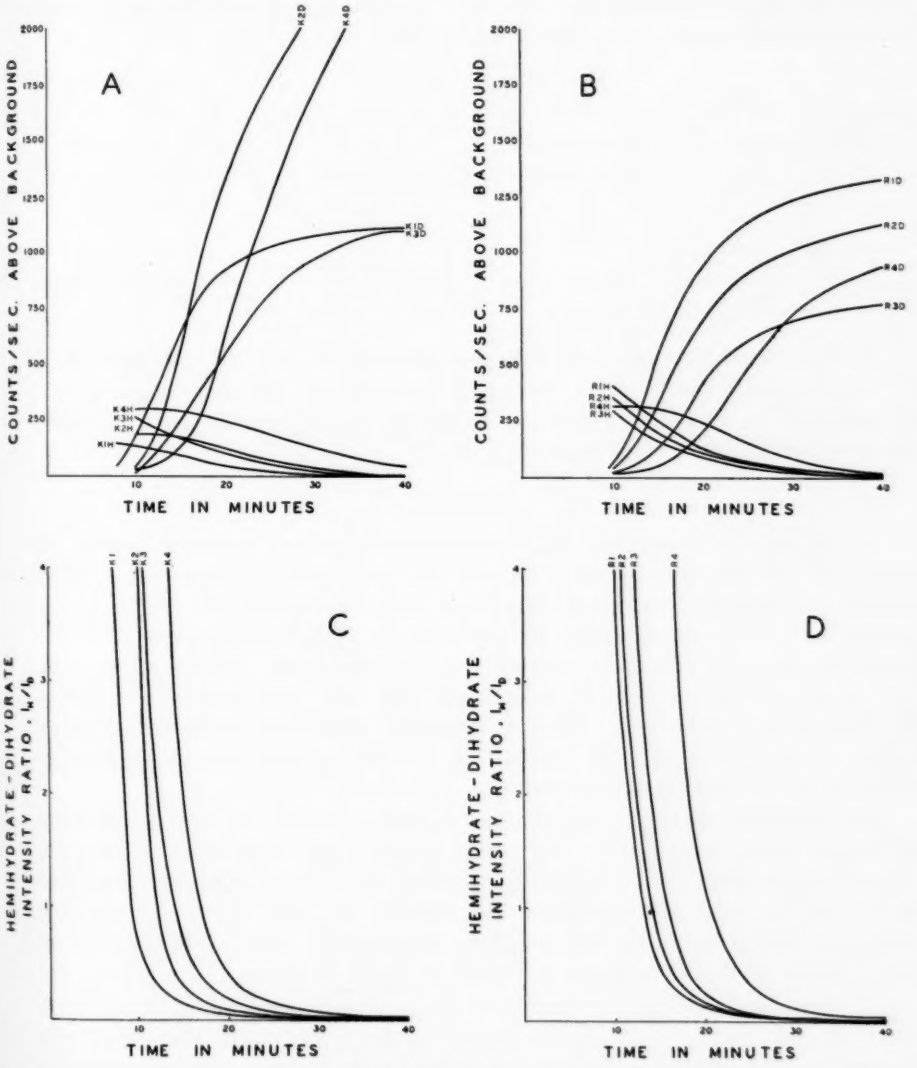

Fig. 3.—Hydration curves for Kerr's Cristobalite Investment and Ransom & Randolph Hygroscopic Investment. (See legend in text and in Table I.)

These values correlate in relative position with the experiments where R & R hygroscopic casting investment was used with the addition of 2.5 ml.

of liquid (Fig. 5) and with values obtained by the addition of 1 ml. of liquid to the R & R investment (Fig. 4). Dimensional changes of the same order of magnitude were obtained using R & R Hygroscopic Investment No. 2.

Addition of 1 ml. of 100 per cent, 10 per cent, and 1 per cent ethyl alcohol in water resulted in increasing expansion values for decreasing alcohol concentration, as illustrated in Table II. Surface tension and viscosity values for the alcohol solutions are also listed in Table II.

TABLE II

ETHYL ALCOHOL (PER CENT)	1-HOUR EXPANSION (PER CENT)	SURFACE TENSION AT 30° C. (DYNE/CM.)	VISCOSITY AT 30° C. (CENTIPOISE)
100	1.4	21.5	1.03
10	1.8	45.9	1.16
1	1.9	66.1	
1 ml. water added	2.0	71.2	0.8

One-hour hygroscopic expansion values for Kerr's Cristobalite dental casting investment upon addition of 1 ml. of alcohol of different concentrations, and upon addition of water. Surface tension and viscosity values for the different liquids are shown.

It was found that the weight of specimens with 1 ml., 2.5 ml., and 3.5 ml. of water added after 48 hours' storage in air would be the same as the weight of specimens to which no water was added during the setting period (weight for all specimens after 48 hours, 53.5 Gm.).

DISCUSSION

It should be recognized that the expansion values reported cannot be considered as effective expansion values for dental casting procedures, since part of the expansion recorded takes place before the initial set of the investments. It is difficult to determine the time at which setting or hygroscopic expansion becomes effective. Factors such as wax and investment strengh, rate of temperature change in investment and wax, and shape and bulk of the wax pattern necessarily affect the time at which the expansion becomes effective. In this study, no attempt was made to determine expansion as related to the actual casting procedure.

As illustrated in Fig. 5, hygroscopic expansion can be obtained by addition of liquids other than water. The term "hygroscopic expansion" is actually a misnomer, at least for this type of expansion but, since it has gained general acceptance, it is used throughout this report to designate that increased expansion which occurs in dental casting investments when allowed to set in the presence of liquid, whether or not this liquid is water.

It was observed that the sharp rise in expansion upon addition of liquid occurred just prior to the loss of gloss and continued until the liquid was absorbed (18 to 30 minutes after start of mix), after which time only a minor expansion similar to the normal setting expansion took place.

The expansion values and curves obtained by the addition of different quantities of water and of other liquids also indicate that the hygroscopic expansion occurs before the normal setting expansion.

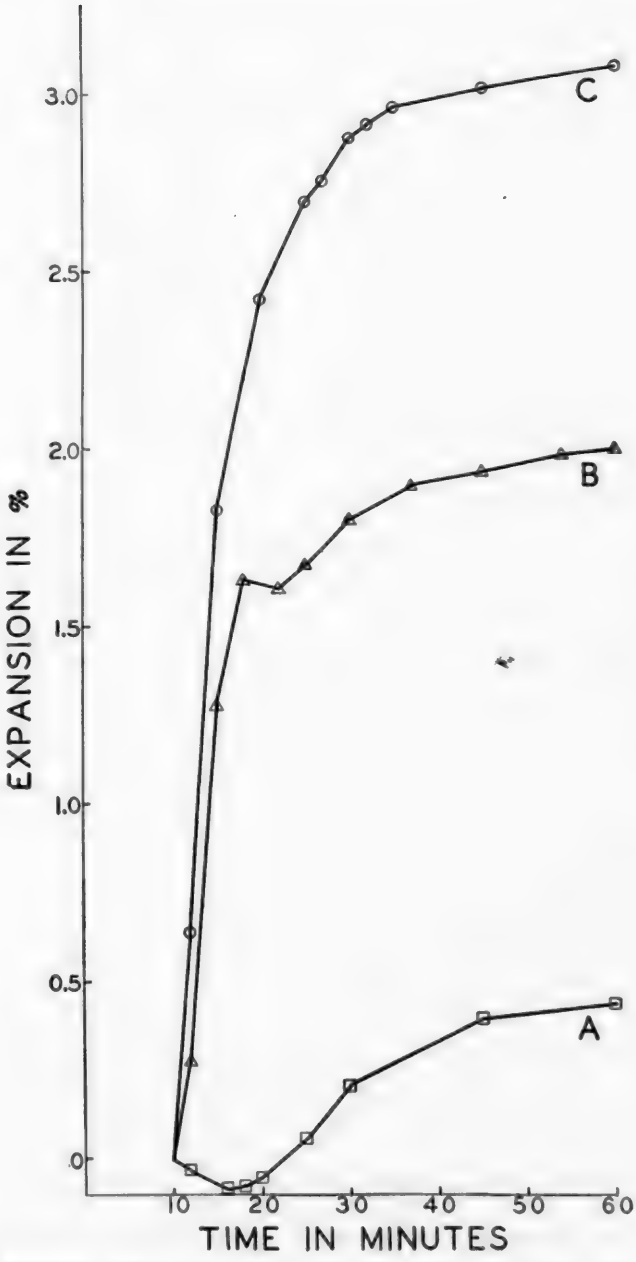

Fig. 4.—Expansion curves for R & R hygroscopic casting investment: *A*, normal setting expansion; *B*, expansion after addition of 1 ml. of water; and *C*, after addition of 2.5 ml. of water.

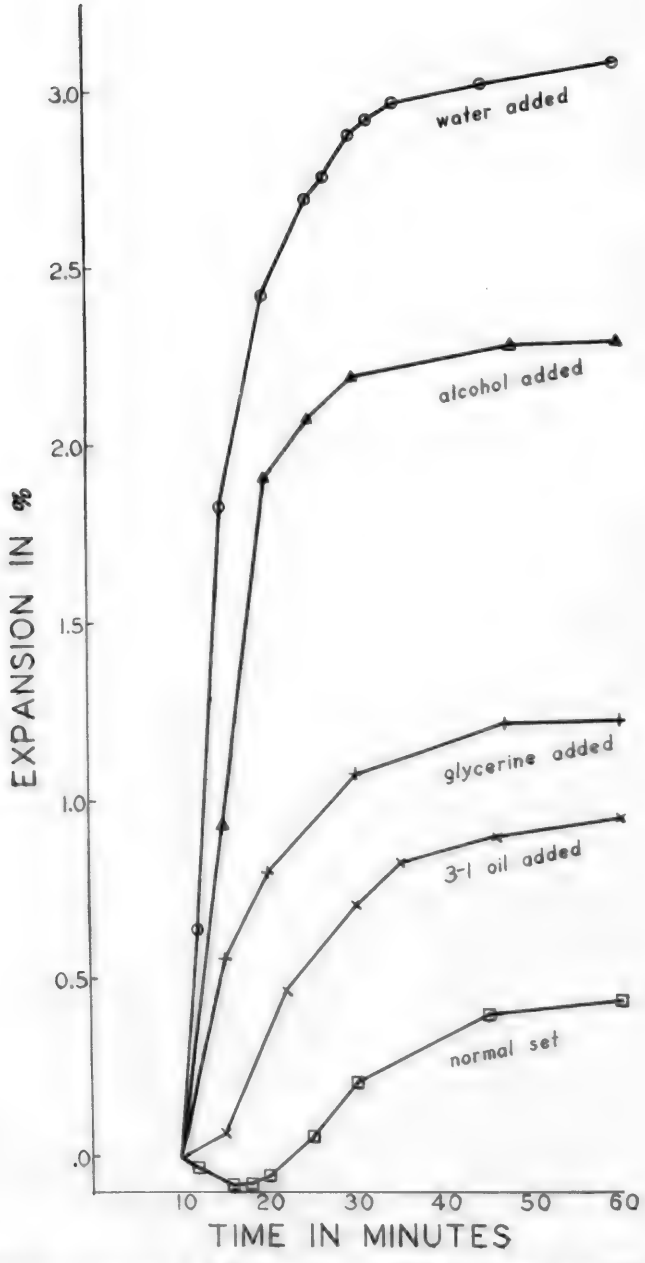

Fig. 5.—Expansion curves for R & R hygroscopic casting investment as affected by addition of 2.5 ml. of water, alcohol, glycerine, and 3-in-1 oil.

All x-ray diffraction data obtained show that the availability of excess water or other liquids during the setting period decreases the final intensity of the dihydrate lines, as compared with the normal setting reaction. The hydration is retarded by addition of water and other liquids during the initial setting period, and by contact with both dry and wet asbestos.

The x-ray diffraction data, dimensional change determinations, and weight determinations indicate that the theory of further hydration is not tenable.

It seems logical, therefore, to attribute the mechanism of hygroscopic expansion to physical factors such as absorption, adsorption, capillary action, and reduction of pressure within the investment due to hydration (reduction of true volume[8, 13]).

The variation in expansion obtained by the addition of: (a) different quantities, (b) different concentrations of the same liquid, or (c) the same quantity of different liquids may be attributed to several factors, such as surface tension and viscosity of the liquids, their effect on crystallization[14] and crystal habits, and solubility of the hemi- and dihydrate in the liquids.

CONCLUSION

On the basis of the evidence presented it is concluded that the phenomenon of hygroscopic expansion, resulting from the presence of excess water or other liquids during the initial setting period, is caused by absorption of liquid into the investment by the reduction of the true volume.

It is believed that the hygroscopic setting expansion occurs as follows: the excess liquid is absorbed by capillary action into the minute interstices as these are formed throughout the investment during the crystallization process. The liquid increases the thickness of the surface film on both the particles of inert material and on the gypsum crystals. As the film thickness is increased, the crystals and particles are forced apart, thus producing expansion of the semisolid mass. After the film thickness has increased and the resulting expansion has taken place, the crystallization continues as under normal setting conditions, with the difference that the crystal interlocking and the expansion due to crystal thrust become less effective because of the larger intercrystalline distances. A liquid with a high surface tension will tend to produce a larger increase in film thickness than a liquid with a low surface tension; consequently, the former will force the particles further apart and produce more expansion.

Under normal setting conditions, air is being absorbed into the investment during the period when the true volume is reduced due to the hydration reaction.

Under hygroscopic setting conditions the liquid covering the surface prevents the absorption of air into the setting investment. Instead, the liquid is pulled in due to the reduction of the true volume.

Other physical phenomena may act as regulating factors for the proposed mechanism. The slight increase in hygroscopic expansion observed by immersion in water of higher temperature (Degni,[6] Landgren and Peyton[7]) may be explained by the fact that the viscosity of water decreases (the fluidity

increases) by increase in temperature. The fact that dihydrate is less soluble in alcohol than in water may be contributing to the effect of alcohol in different concentrations. Also, alcohol is known to modify crystal habits.

The mechanism here proposed will also account for observations reported by other investigators, including decreased particle size of the inert material causing an increase in hygroscopic expansion (Scheu,[2] Docking[5]) because the total internal surface is larger; higher investment/water ratio causing increased hygroscopic expansion (Degni;[6] Landgren and Peyton;[7] Lyon, Dickson, and Schoonover[8]) because the material is denser (more particles per unit volume); inert material, difficult to wet, causing less hygroscopic expansion than others (Lyon, Dickson, and Schoonover[8]) because particles difficult to wet decrease capillary action; and decreased compressive strength resulting from the investment setting under hygroscopic conditions (Skinner[15]) because the crystal interlocking is less effective due to the expansion, and because there is less material per unit volume than after normal setting.

SUMMARY

1. Hygroscopic expansion can be produced by liquids other than water. 2. X-ray diffraction data show that hygroscopic expansion is not caused by hydration. 3. Hygroscopic expansion is explained on the basis of physical characteristics of the materials involved.

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SURFACE APPEARANCE OF TOOTH CAVITY WALLS WHEN SHAPED WITH VARIOUS INSTRUMENTS

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SINCE the introduction of faster operating speeds for rotating instruments, there has been an increased concern regarding the effects of various instruments on the cut tooth surface. Very little information on this subject exists in the literature. The work of Street[1] describing the effects of instruments on enamel walls represents the most complete report in the current literature.

It is the purpose of this report to describe an alternate procedure for examining the tooth surface as it exists after being cut with various instruments. Particular emphasis will be placed upon the technic and procedure of observing the cut tooth surface.

ROTATING INSTRUMENTS

It has seemed desirable not only to determine the appearance of the freshly cut tooth surface but also to examine the surface appearance of the cutting instrument. Fig. 1 shows the surface appearance of 6 inverted cone type diamond instruments. Some difference in surface texture and appearance of these instruments can be detected. Some have relatively sharp corners while others are rounded, and some display quite uneven applications of the diamond particles while others appear to have a more uniform surface texture. In Fig. 2 is shown a group of cylindrical or taper type diamond instruments. These also show a lack of uniformity in surface appearance and details of the instrument. The small disks represented in Fig. 3 show a lack of uniformity with some variation in surface roughness. The disks in Fig. 4 represent the large diameter disks suitable for the slice type of preparation, and show some evidence of different particle size and surface texture of the cutting instruments. Interesting comparisons might be made with carborundum stones and various types of dental burs, but space does not permit inclusion of all the rotating instruments in this presentation.

TECHNIC FOR SAMPLE PREPARATION

Because of the translucent nature of tooth tissue, it is difficult to examine microscopically and to record photographically the surface appearance of the freshly cut tooth by means of reflected illumination. Street used lamp black paste which was wiped on the cut surface and, after drying, the excess material was removed leaving the scratches from the instrument filled with the lamp black.

This study supported by a contract between the United States Air Force, School of Aviation Medicine, Project No. AF 16 (600) 427, and the University of Michigan, School of Dentistry.

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In order to render the tooth more opaque in this study the tooth surface has been shadowed with a thin deposit of copper in the same way that shadowing is used to develop the detail of the replica technic for high power light microscope and electron microscope studies. The procedure of proper deposition was identical to that ordinarily employed for the replica technic in which a copper filament is vaporized in vacuum permitting the copper to be deposited on the surface of the specimen. The illustrations which are used in this report represent teeth that were placed in the vacuum chamber and had the copper deposited directly on them rather than on a replica of the tooth surface. The technical details of the operation of shadow deposition have been adequately described in the literature.[2] The thickness of the metal deposit can be controlled by the time and condition of deposition. In these studies, the deposit was not sufficiently thick to alter the surface appearance resulting from the action of the cutting instrument, but was sufficient to render the surface somewhat opaque and permit better microscopic examination.

Fig. 1. Fig. 2.

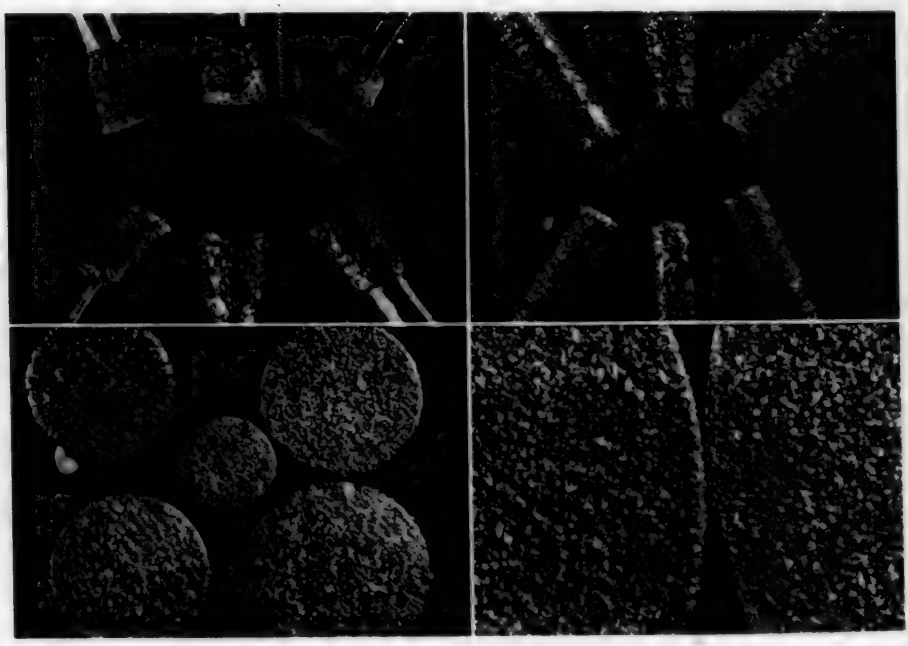

Fig. 3. Fig. 4.

Fig. 1.—Representative inverted cone diamond instruments.
Fig. 2.—Cylindrical and taper type diamond instruments.
Fig. 3.—Variations in surface texture of small diamond disks.
Fig. 4.—Two representative large size diamond disks.

Freshly extracted teeth were chosen and were kept wet until the cavity was shaped with the various instruments chosen. Studies were made using a variety of diamond instruments at both high (12,000 r.p.m.) and low (4,000

r.p.m.) speeds of operation. A variety of carborundum disks were used at both high and low speeds in the dry condition while diamonds were operated both wet and dry. The surface developed by the use of an enamel hatchet has been included. Photographs of the cutting instruments and the surfaces developed on the cut tooth have been recorded at relatively low magnifications of approximately 20 diameters. Some details seem to be lost because of lack of depth of focus when higher magnifications are employed and lower magnifications do not reveal the full surface appearance.

SEPARATING DISKS

An interesting surface results from the application of a "Joe Dandy" type of disk to slice the tooth as shown in Fig. 5. Such a disk operated dry at slow speed develops a series of symmetrical scratches, some of which appear to be much deeper than others. While the surface appears to be uniformly abraded, it cannot be interpreted as representing a smooth surface. In Fig. 6 is shown the surface developed by the same disk, when operated wet and at fast speeds. While this illustration represents a contour cut of the tooth rather than a straight slice, the cut surfaces are similar in appearance with respect to the disk action on the tooth. There is no pronounced evidence of a different surface appearance resulting from the same instrument at different speeds, or when used wet or dry. The surface developed by a so-called "lightning disk" is shown by Fig. 7. This represents a slice comparable to that of Fig. 5. The disk was operated dry at a slow speed for this cut. While the cut resembles closely that made by the "Joe Dandy" disk in general appearance, there is perhaps a less pronounced and less well-defined scratch developed by this disk. Possibly this is related to the different texture of the disk.

DIAMOND DISKS

In general, it appears that the surfaces resulting from the action of diamond disks have scratches that are a little coarser than those from corresponding carborundum disks (Fig. 8). This represents the surface cut with a popular diamond disk operated wet and at high speeds. It is apparent that the operator changed the position of the instrument during the cut but it reveals also that coarse particles on the disk left deep scratches. The surface left by the action of another product when operated wet at slow speeds is shown in Fig. 9. This surface is comparable to that of Fig. 8, and shows no significant result of the slower speed of operation.

Some dentists claim it is desirable to refinish the surface resulting from the diamond disk with a small diameter, cylindrical, or tapered carborundum stone. Such an operation is described as "honing" the surface. In Fig. 10 is shown a portion of a tooth which was first sliced with a diamond disk, after which the portion on the right was finished with a No. 28 pointed white stone of small diameter. The surface appearance of the two areas is quite different with the surface scratches less pronounced where the stone was used, although this surface presents a lack of uniformity due to the uneven action of the small diameter stone.

Fig. 5.

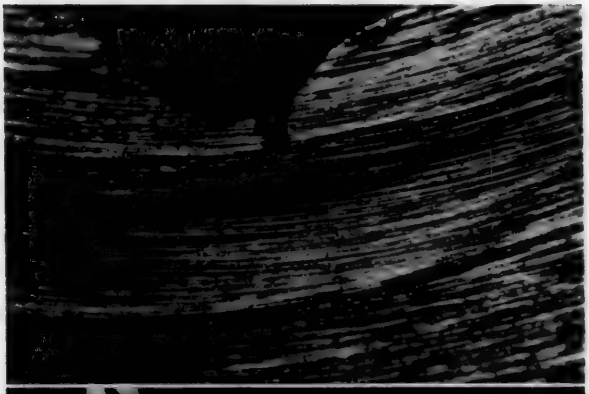

Fig. 6.

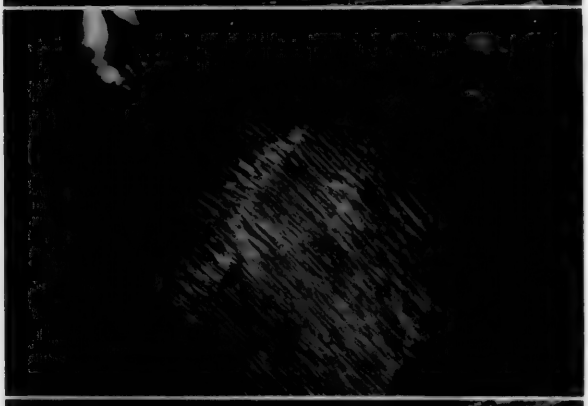

Fig. 7.

Fig. 5.—Tooth surface left from action of "Joe Dandy" disk operated slowly and dry.
Fig. 6.—Result of cutting tooth with "Joe Dandy" disk while wet at fast speed.
Fig. 7.—Cut surface resulting from use of lightning disk dry at slow speeds.

Fig. 8.

Fig. 9.

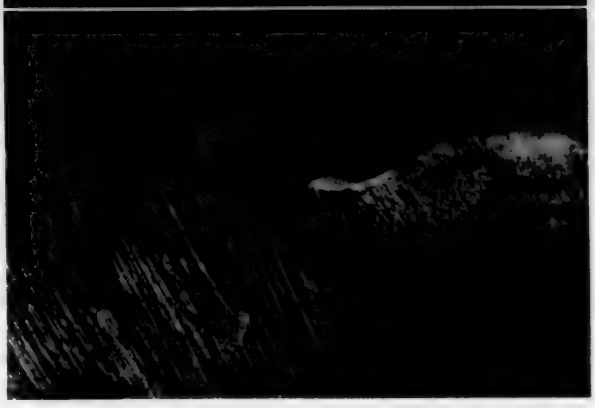

Fig. 10.

Fig. 8.—Surface produced by diamond disk operated wet at high speeds.
Fig. 9.—Surface produced by diamond disk operated wet at slow speeds.
Fig. 10.—Surface resulting from application of diamond disk at left and carborundum stone at right.

Fig. 5.

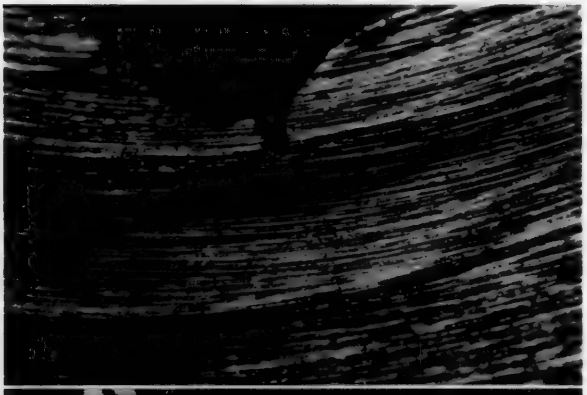

Fig. 6.

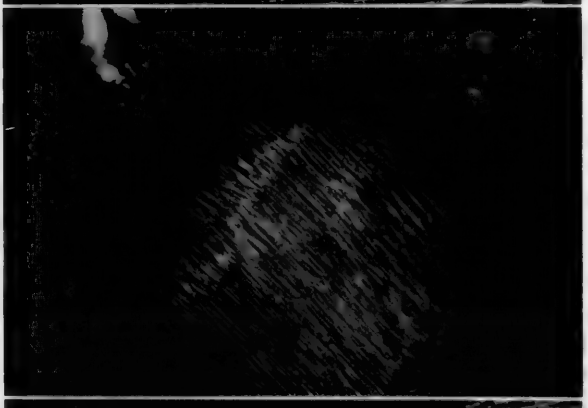

Fig. 7.

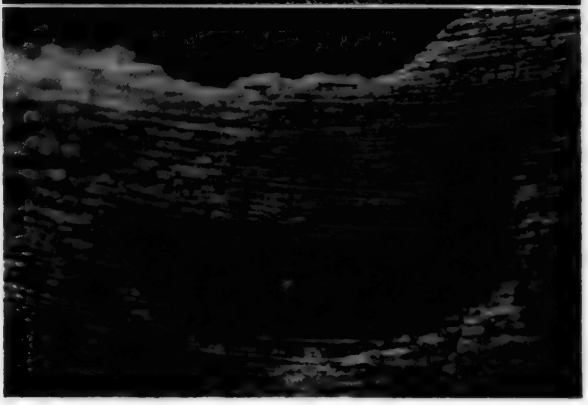

Fig. 5.—Tooth surface left from action of "Joe Dandy" disk operated slowly and dry.
Fig. 6.—Result of cutting tooth with "Joe Dandy" disk while wet at fast speed.
Fig. 7.—Cut surface resulting from use of lightning disk dry at slow speeds.

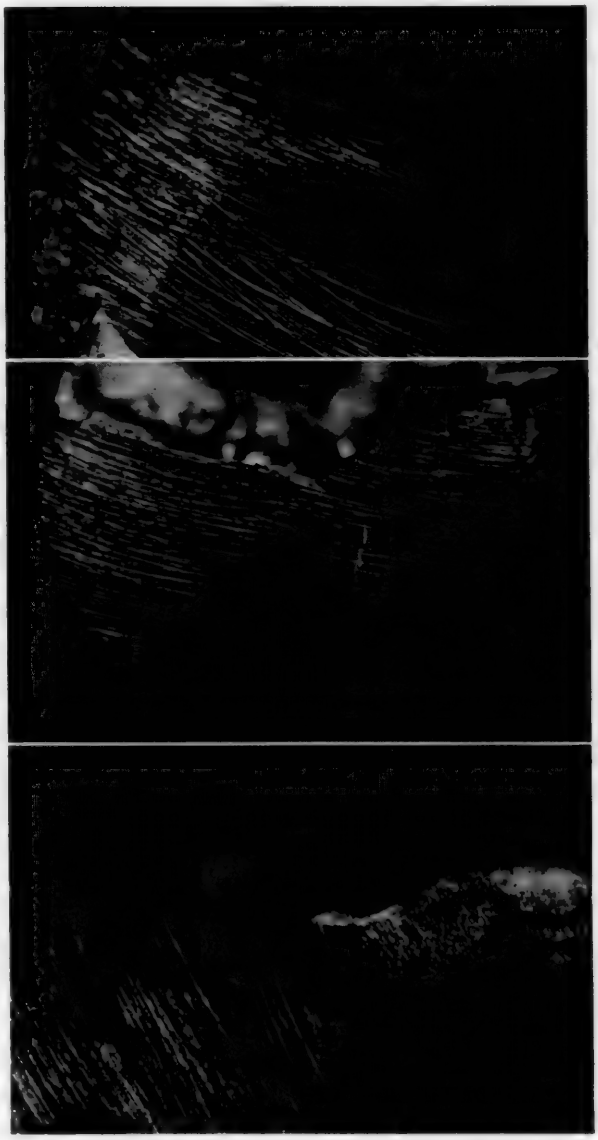

Fig. 8.

Fig. 9.

Fig. 10.

Fig. 8.—Surface produced by diamond disk operated wet at high speeds.
Fig. 9.—Surface produced by diamond disk operated wet at slow speeds.
Fig. 10.—Surface resulting from application of diamond disk at left and carborundum stone at right.

NO. 560 DIAMONDS

In the shaping of certain cavities it is often desirable to employ a straight fissure type diamond instrument No. 560, of a small diameter. In size and shape this instrument resembles the No. 59 straight fissure bur. When applied dry at slow speeds to the tooth structure, there results a surface appearance which lacks uniformity both with respect to the depth of the surface scratches and also from one area of the tooth to another.

Fig. 11.

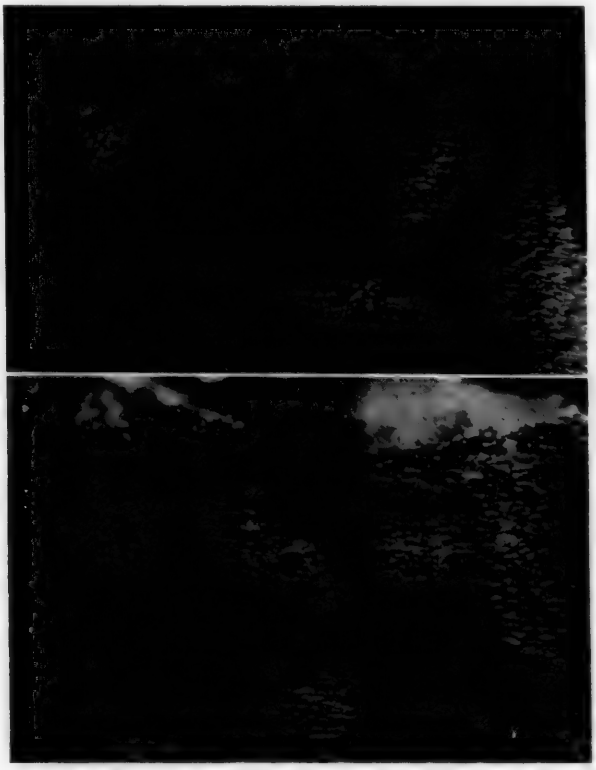

Fig. 12.

Fig. 11.—Appearance of tooth after being cut with No. 560 type diamond when operated wet at slow speeds.

Fig. 12.—Appearance of tooth after being cut with No. 560 diamond when operated wet at high speeds.

When using the same instrument wet at slow speeds the resulting tooth surface is shown in Fig. 11. This surface resembles closely those resulting from using the instrument dry and also shows the effect of the irregular diamond particles in the instrument. In Fig. 12 is the surface developed from this same instrument operated wet at high speeds. From these observations there seems to be no evidence to indicate that a different tooth surface results from either

different operating speeds or with the presence or absence of water during the cutting operation. While the surface from the small diameter diamond resembles in some ways that of a small diameter carborundum stone, it is generally filled with deeper scratches from a diamond instrument than from a similar carborundum instrument.

DENTAL BURS

By contrast with the diamond and carborundum instruments the surface resulting from the action on the tooth of a No. 558 steel cross-cut fissure bur when operated dry at slow speeds, is shown in Fig. 13. The surface presents

Fig. 13.

Fig. 14.

Fig. 13.—Relatively smooth surface of tooth resulting from action of No. 558 steel cross-cut fissure bur.

Fig. 14.—Surface of cut tooth resulting from action No. 58 straight fissure bur.

less evidence of deep scratches than with the diamond but shows a nonuniformity due to the uneven cutting action from one area to another. No significant differences could be detected when the bur was operated fast or slow, or when wet or dry. The surface resulting from a No. 702 cross-cut taper fissure bur is essentially the same as that from the No. 558 bur.

When using a No. 58 steel straight fissure bur dry at low speeds, the surface developed is similar to that shown in Fig. 14. This surface represents perhaps the smoothest and most uniform one observed with small diameter rotating instruments. A similar surface results from a No. 601 steel bur which is similar in design except for a taper shape. The surface here resembles that from a planing instrument and lacks the deep scratches characteristic of diamonds and stones. Again, a change in the speed of operation did not change the surface appearance developed on the tooth by the bur.

Fig. 15.

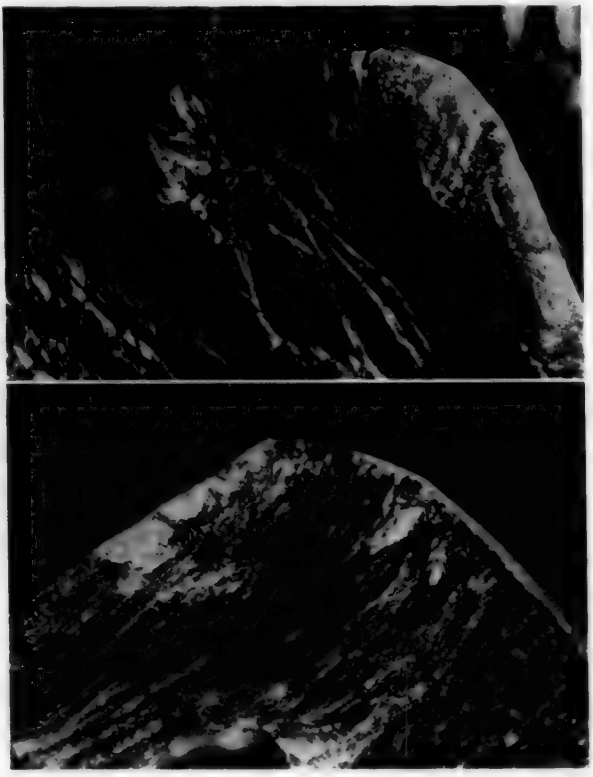

Fig. 16.

Fig. 15.—Cavity wall resulting from cut by enamel hatchet.
Fig. 16.—Appearance of cavity wall shaped with enamel hatchet.

HAND INSTRUMENTS—ENAMEL HATCHET

Some operators have expressed the opinion that the hand instrument develops the best or smoothest surface on the cavity wall. In Fig. 15 is shown the surface resulting from the use of an enamel hatchet. This shows a lack of uniformity over the area and perhaps illustrates also that the skill of the operator in the use of the instrument is an important factor in developing a good sur-

face in the cavity. In Fig. 16 is shown another cavity wall shaped with the same instrument. While some improvement is shown in the surface condition of the cavity wall, it is less smooth than that resulting from a fissure bur. The accessibility of the cavity to the application of the instrument may modify to some degree the results of different operators, but in general the hand instruments do not seem to present a superior surface to that resulting from rotary instruments. This agrees in general with the observations of Street.

SUMMARY

A method suitable for examining the cavity walls of the extracted tooth has been presented. This method consists of shadowing the tooth surface with a thin film of copper or other metal to make the tooth less translucent. This operation permits the examination of the cut surface by reflected light at various magnifications.

Results are presented from the use of different cutting instruments to cut tooth tissue. Little difference in results can be observed from different speeds of rotation, or from using the instrument wet or dry, when the cut surface is examined macroscopically. A difference between burs, carborundum stones, and various diamond instruments is evident.

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FLUID PENETRATION BETWEEN FILLINGS AND TEETH USING CA^{45}

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THIS paper is intended to be a follow-up study on earlier experiments to determine the fluid penetration around amalgam and gold fillings that were placed in green teeth. The results of those earlier experiments seemed to indicate that amalgams produced the least marginal penetration.

In this study 12 teeth, which were extracted at the University of Minnesota during the summer of 1953, were used. The teeth all contained amalgam or gold restorations which had been in use by patients for varying lengths of time up to seven years. Upon extraction the teeth were stored in a solution of physiological saline and thymol at 6° C. until the 12 were collected.

The radiocalcium* used was in the form of calcium chloride in a weak hydrochloric acid solution that was neutralized. This solution was used with a neutral normality to avoid any possible dissolution or weakening of the tooth structure by acid. The specific activity of the material was 80 microcuries per gram calcium. The concentration of Ca^{45} used was approximately 1 million counts/min./c.c. according to our counting methods. This was approximately 6.76 mg. Ca/c.c.

The roots of the teeth which had been stored in physiological saline solution were covered with a plastic coating (fingernail polish) and the crowns were immersed in the neutralized solution containing Ca^{45} for 24 hours. When removed from the solution, the teeth were washed and brushed under running distilled water to eliminate any radiocalcium that had not penetrated the tooth. The teeth were then embedded in methyl methacrylate resin and sectioned through the fillings. The exposed surfaces were ground smooth, and the sections were placed on film.

Much time has been spent preparing radioautographs on various sensitized films and plates to find the most suitable type emulsion for this work. Of the various nuclear track plates available from Eastman, the most satisfactory for this work seems to be the Kodak Nuclear Track Plates type NTB-2 with 10 micron emulsion thickness and an anti-abrasive coating. This type of plate gives a very clear image without background fog if it is used in complete darkness. Its main advantage is that, because of its extremely small grain size, an enlarged print can be made from it or it can be observed under a microscope without loss of definition.

*The radiocalcium used was obtained from the Oak Ridge National Laboratory on allocation from the United States Atomic Energy Commission by Dr. Armstrong, head of the University of Minnesota's Department of Physiological Chemistry and was furnished under supervision for this investigation.

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It does have quite a few disadvantages, however. It is available only on special order from Eastman, its shelf life is very short, and probably its greatest disadvantage is the long exposure time necessary. Thirty- and forty-five-day exposures are not unusual and since the only way to establish the necessary exposure time for any particular need is by trial, using this plate can be a very time-consuming procedure.

For this type of study it is suggested that Eastman No-screen X-ray film be used. It is cut to the size of small supporting pieces of masonite, pressed wood, or any stiff material about 2½ by 3½ by ¼ inches. If the specimen is bound to this support with rubber bands having the film sandwiched between, movement of specimen or film during the exposure time is eliminated, which ensures sharp outlines on the radioautograph. This film is easier to work with, will need a much shorter exposure time (from 16 to 66 hours, usually), and for most purposes will give a very satisfactory radioautograph.

Specimens and film were placed in a metal container and set aside during the exposure time after the top was sealed with photographic tape.

Since Ca^{45} is a soft emitter with an average beta particle energy of 0.26 Mev and has no gamma radiation, very thin materials, such as a pyrex beaker, will be a shield against the radiation. Therefore, no extreme precautions are necessary to guard against radiation damage. Ordinary precise laboratory procedures can be safely followed. However, all pipetting must be done with automatic pipettes, a steel workbench top is used so any spill can be wiped up, and all waste is kept for 6 months before it can be safely disposed of in an incinerator.

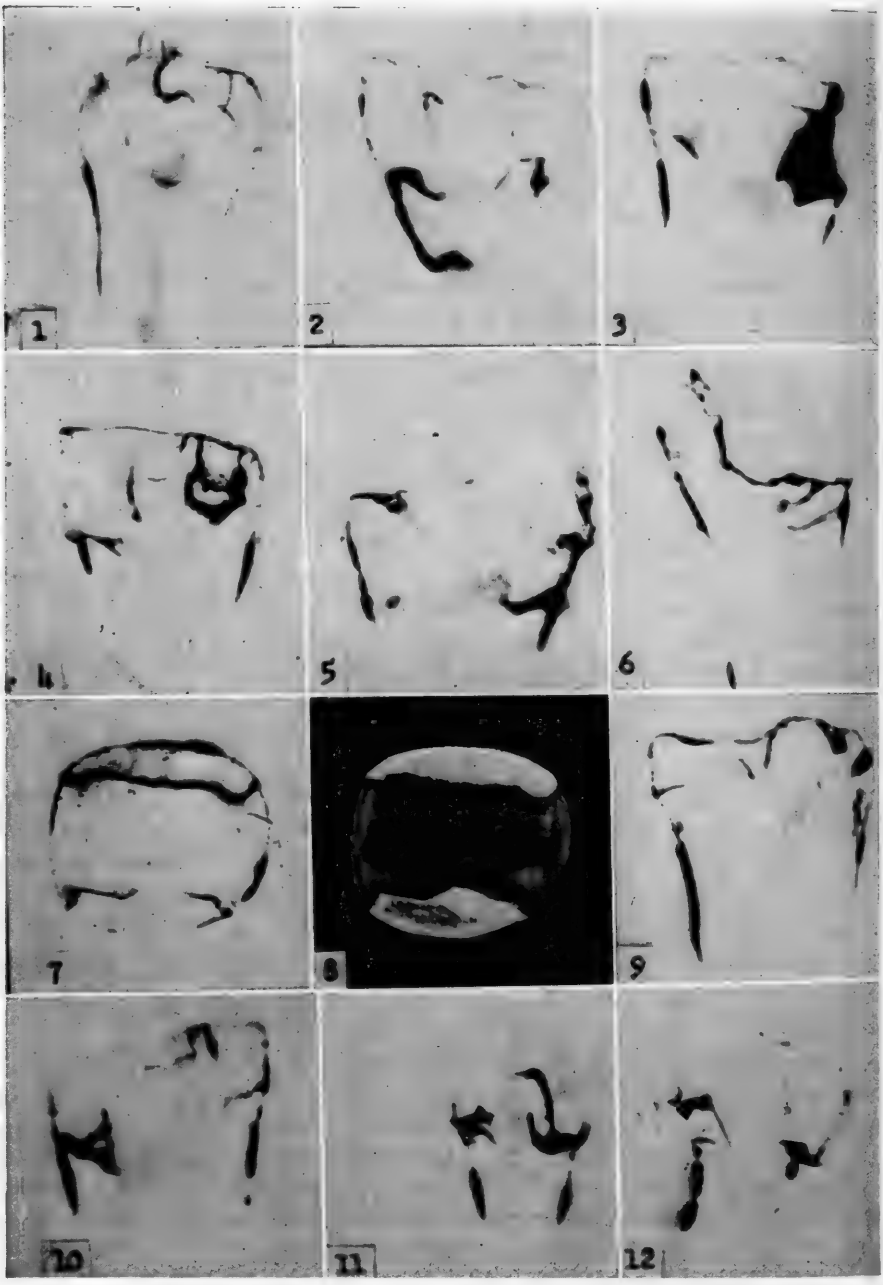
AUTORADIOGRAPHS

Fig. 1 is a section through the lingual side of a lingual occlusal amalgam filling. The filling was about 4 months old at the time of extraction. Penetration of solution carrying Ca^{45} is readily apparent, particularly toward the mesial where there is evidently some injury of the enamel at the margin of the filling.

Fig. 2 shows a large MOD inlay in an upper second molar. There was penetration entirely around and underneath this filling, particularly at gingival margins.

Fig. 3 shows a mesial occlusal amalgam filling placed in an upper first molar showing very little penetration of the Ca^{45}. The tooth was lost due to a carious lesion on the distal side of the tooth.

Fig. 4 is a section through 2 amalgam fillings on an upper second molar. Penetration around the amalgam filling on the mesial occlusal is only slight. The penetration around the buccal occlusal amalgam filling is interesting in that it shows a tremendous penetration around the cement base under the amalgam. This is the usual type of penetration found around cement fillings or cement bases. Part of the amalgam filling was broken out behind this area so the Ca^{45} could enter the cement there quite easily.



Figs. 1-12.—*(For legends, see opposite page.)*

Fig. 5 is a section through mesial occlusal inlay placed Jan. 24, 1947. The tooth was extracted on June 8, 1953. This seems to have been quite a good restoration as far as penetration is concerned since a longer than average exposure was necessary to show this penetration.

The filling shown in Fig. 6 was an amalgam in a lower second molar. The tooth was an extremely poor risk at the time of the filling, and was extracted only several weeks after the insertion of the filling.

Fig. 7 is a radioautograph of a section through the gingival portion of a lower second molar showing adaptation of a gingival buccal and a gingival lingual filling. The fillings were about three years old, and there were recurrent caries around the gingival lingual filling shown at the bottom of the illustration. Fig. 8 is a photomacrograph of the same section.

Fig. 9 is a section through a lingual occlusal amalgam filling in an upper first molar. The filling is obviously well adapted and shows only a small amount of penetration.

Fig. 10 shows a mesial occlusal distal amalgam filling showing quite extensive penetration at the gingival on the mesial. There was a considerable amount of penetration around the cement base.

Fig. 11 is of an MOD inlay placed about seven years before the tooth was extracted to prepare the mouth for full dentures. Both the mesial and distal gingival steps show rather large amounts of penetration.

Fig. 12 shows an MOD gold inlay in an upper second bicuspid. The history is the same as for the inlay in Fig. 11.

The obvious question must be asked—Why do fillings last as well and as long as they do with the apparent ease of fluid penetration around them? What takes place between the teeth and the fillings to prevent recurrent caries?

SUMMARY

An earlier study using radioactive calcium and fillings of various types in green teeth showed obvious penetration around all types of fillings. Speculation on this finding would naturally lead one to wonder why fillings perform as well as they do for long periods of time when it is obvious solutions penetrate between the filling and the teeth quite easily. The purpose of this study

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Fig. 1.—Lingual side of LiOamalgam.
Fig. 2.—Buccal side of MOD amalgam.
Fig. 3.—Buccal side of MOLi amalgam.
Fig. 4.—Cement liner under amalgam on right.
Fig. 5.—MO inlay.
Fig. 6.—Amalgam placed temporarily. Tooth was to have been crowned.
Fig. 7.—Bu amalgam. Lingual filling had recurrent caries.
Fig. 8.—Photomacrograph of tooth section.
Fig. 9.—Amalgam fillings, tooth believed to be gangrenous.
Fig. 10.—MOD amalgam.
Fig. 11.—Gold inlay.
Fig. 12.—Gold inlay.

was to recover fillings that had been in service to determine whether or not solutions would penetrate around these fillings after they had been in service in the oral cavity as readily as around the fillings placed in green teeth. It can be concluded, at least from these radioautographs, that service in the oral cavity has certainly not made these fillings less susceptible to fluid penetration.

IN VITRO CULTIVATION OF RAT MOLAR TOOTH GERMS. II

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THE period of development, reported to date, limits the use of the in vitro cultivation of tooth germs as an experimental method.[1-9] Variations in technic and media are presently under investigation to establish a procedure which will provide a longer period of development.

The Carrel flask[10] presents a versatile method which, in exploring the predilection of organized growth, lends itself to effective pursuit of the problem. One variation in technic is to plant the tooth germ either "in" or "on" the plasma coagulum. The growth, development, and survival of "in the clot" cultivation, which has already been reported,[9] may be compared with the present study of "on the clot" cultivation which permits the explant to be exposed to the supernatant nutritional fluid without its diffusing through the clot. Explants cultured "on the clot" exhibited greater development of the ectodermal elements, for the same period of cultivation, than occurred in "in the clot" studies.

MATERIALS AND METHODS

Forty-seven tooth germs were cultivated "on the clot" to determine growth, development, and survival for comparison with "in the clot" cultivation using similar explants and media.[9]

All explants, dissected from nineteen-day rat embryos, were derived from Rockland albino rats.[9-11] The tooth germs contained the enamel organ, dental papilla, and follicular wall. Chicken plasma was obtained in the frozen state. Tyrodes solution and rat embryo extract were prepared as previously described.[10] The 1 c.c. coagulum used for each explant consisted of 3 parts chicken plasma and 1 part of 25 per cent rat embryo extract.[10] The 1.5 c.c. supernatant fluid contained 1 part of 25 per cent rat embryo extract and 3 parts of Tyrodes solution.

The tooth germs, dissected from 3 litters of embryos, were planted "on the clot." The supernatant fluid and the plasma coagulum contained .005 per cent phenol red and were adjusted to pH 7.6. No transfers were made. At four-day intervals, all cultures were washed with Tyrodes solution for two hours and fresh supernatant fluid was added. Observations were made every four days and the explants were photographed at that time. Specimens were taken at intervals of four days to record growth, development, and survival. The maximum period of culture was twenty days for comparison with previous

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results of "in the clot" culture. Seven explants were cultured for four days each, 16 for eight days, 14 for twelve days, 7 for sixteen days, and 3 for twenty days. The histologic technic was similar to that previously used.[9]

RESULTS

Observations made during cultivation were concerned with growth, morphology, translucency or opacity of the explant, and outgrowth characteristics. Cultivation was terminated at four-day intervals up to twenty days for histologic examination, which provided additional information on survival, differentiation, growth, and development. These findings, correlated with a table of development in vivo,[12] established the amount of development obtained in vitro.

GROSS OBSERVATIONS

Immediately upon planting, the tooth germs presented an opaque, egg-shaped mass. Twenty-four hours later they became sufficiently translucent to permit recognition of the tooth germ elements. At four days the translucency permitted photography of growth and development (Figs. 1, 2, and 3).

After sixteen days, the tooth germs deviated from the usual pattern, became opaque and no further gross observations of the elements were possible (Figs. 1, *E* and 2, *E*).

Observations of growth were made exclusive of the outgrowth. In all cases, there was an increase in size of the original explant up to sixteen days (Figs. 1, 2, and 3). The rate of growth was most rapid during the first eight days, diminishing thereafter. At sixteen days, when the explant became opaque, it also diminished in size over the maximum growth (Figs. 1, *E* and 2, *E*).

Observations of development depended upon orientation of the tooth germ "on the clot." In most cases it was possible by gross examination to recognize the preameloblast layer, formation of predentin, and the development of cusp outline at the future dentinoenamel junction (Figs. 2, *C* and 3, *A*).

The outgrowth which appeared twenty-four hours after planting consisted mostly of fibroblasts with some epithelium in the area of the severed lamina and neck. At four days, there was profuse outgrowth (Figs. 1, 2, and 3). The rapidly proliferating cells persisted for approximately twelve days, at which time large, round monocyte-like cells appeared in the area (Figs. 2, *C*, 14, and 15). Liquefaction of the clot and cytolysis of the outgrowth followed the appearance of the monocyte-like cells (Figs. 1, *E*, 2, *E*, and 15). The process was progressive; the monocyte-like cells accumulating at the periphery of the digested areas. At twenty days, scattered fibroblasts again radiated from the tooth germ as a new outgrowth (Fig. 1, *E*). The process of liquefaction was limited to the outgrowth and did not involve the tooth germ (Fig. 1, *E*).

In removing the explants from the flasks it was observed that most of the tooth germs had lost their egg-shaped morphology and were biconvex. This phenomenon occurred as early as four days after planting, although in isolated cases the tooth germ retained its normal morphology.

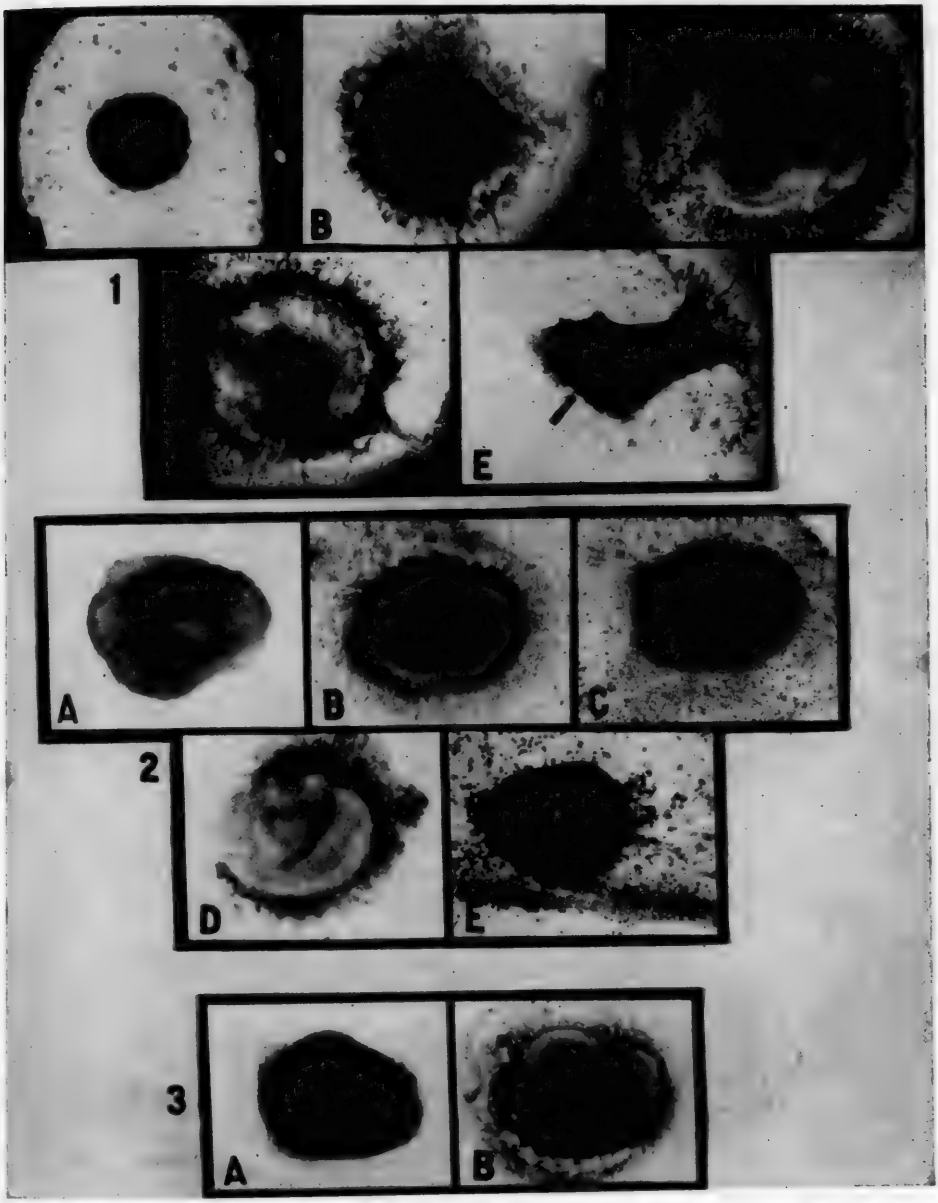

Fig. 1.—Nineteen-day rat embryo tooth germ explant. *A*, one day in flask; *B*, eight days; *C*, twelve days; *D*, sixteen days; *E*, twenty days. Note: Monocytes and liquefaction of clot and outgrowth, *D*. Arrow points to new outgrowth, *E*. Liquefaction did not effect tooth germ. Note opacity of explant, *E*. (Orig. mag. ×35; reduced ⅔.)

Fig. 2.—Nineteen-day rat embryo tooth germ explant. *A*, one day in flask; *B*, four days; *C*, eight days; *D*, twelve days; *E*, sixteen days. Note: Dentin cusp outline, *B*, *C*. Monocytes and opacity of explant, *E*. (Orig. mag. ×35; reduced ⅔.)

Fig. 3.—Nineteen-day rat embryo tooth germ well oriented in flask. *A*, one day; *B*, four days. Note: Cusp outline at dentinoenamel junction. (Orig. mag. ×35; reduced ⅔.)

HISTOLOGIC RESULTS

Fig. 4 illustrates the development of a nineteen-day rat embryo tooth germ after four days' cultivation on the clot. Dedifferentiation of the cells of the outer tunic and the proliferation of fibroblasts into the central cell area of the enamel organ indicates normal development (Fig. 5). Reversal of polarity of the preameloblasts had occurred in the areas of first apposition. The cell-free zone at the periphery of the papilla, and beginning differentiation of odontoblasts at the dentin cusp tips was evident. Differentiation of odontoblasts was incomplete. Four days of in vitro culture produced between one and two days of in vivo development.

Eight days of cultivation (Fig. 6) resulted in a flat specimen in which the dentin cusp outline was obvious, but underdeveloped. The outer tunic had dedifferentiated. The central cell area of the enamel organ was partially occupied with fibroblasts, and the presence of dividing preameloblasts indicates the directional potential toward cusp formation at the dentinoenamel junction (Fig. 7). Differentiation of the stratum intermedium had begun.[12] The presence of the cell-free area and odontoblasts in addition to the development of the enamel organ establish the in vitro development to be equivalent to that found at birth.

Fig. 8 illustrates the development observed after twelve days of cultivation. Despite the flat specimen, the predetermination to achieve cusp formation at the dentinoenamel junction was evident. The outer tunic had dedifferentiated and the presence of fibrous tissue in the central-cell area of the enamel organ suggests postnatal development (Fig. 9). Mitosis of the preameloblasts is active while, in other areas, elongation and the presence of terminal bars may be seen (Fig. 9). The development of the papilla failed to keep pace with that of the enamel organ which showed approximately one day of postnatal development. There was no advance over the result of eight days of culture in the peripheral fibroblasts of the papilla. No degeneration had occurred.

After sixteen days of cultivation, changes in the enamel organ were manifested in the outer tunic, which exhibited dedifferentiation, and replace-

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Fig. 4.—Section of tooth germ cultivated four days. (Orig. mag. ×150; reduced ⅔.)

Fig. 5.—High magnification of Fig. 4. Note the dedifferentiation of outer tunic at upper left. (Orig. mag. ×450; reduced ⅔.)

Fig. 6.—Section of tooth germ cultivated eight days. (Orig. mag. ×150; reduced ⅔.)

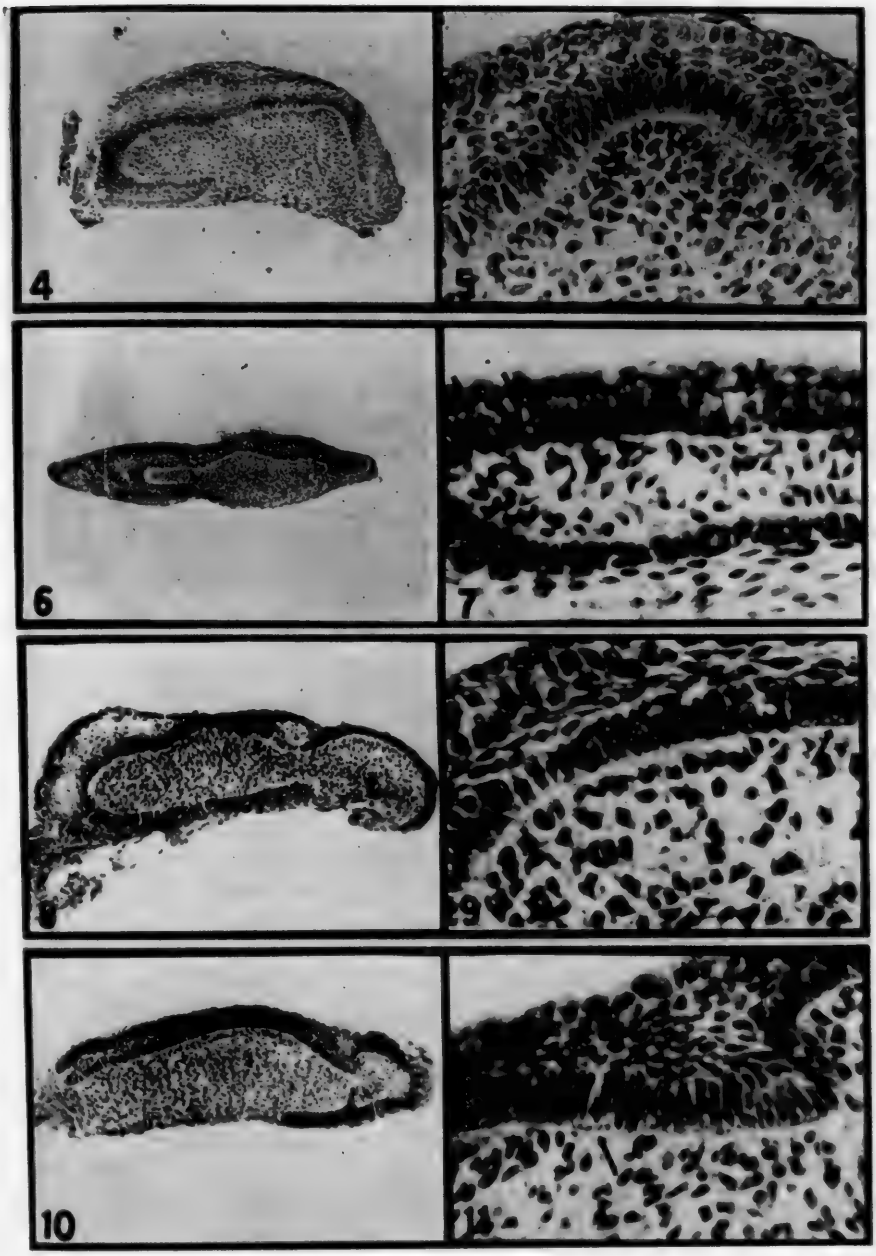
Fig. 7.—High magnification of tooth germ cultivated eight days. Section cut transversely through area of dentin cusp tip at dentinoenamel junction. Arrows point to dividing cells. Note the differentiating odontoblasts at extreme right. (Orig. mag. ×450; reduced ⅔.)

Fig. 8.—Section of tooth germ cultivated twelve days. (Orig. mag. ×150; reduced ⅔.)

Fig. 9.—High magnification of Fig. 8. Note the fibroblasts in central cell area and dividing cells of inner enamel epithelium. Arrow points to terminal bars. Odontoblasts have failed to differentiate. (Orig. mag. ×450; reduced ⅔.)

Fig. 10.—Section of tooth germ cultivated sixteen days. (Orig. mag. ×150; reduced ⅔.)

Fig. 11.—High magnification of Fig. 10. Note the dedifferentiated outer tunic and fibroblasts in central-cell area. Arrow points to terminal bars and Tomes processes. Peripheral fibroblasts are undifferentiated. (Orig. mag. ×450; reduced ⅔.)



Figs. 4-11.—(*For legends, see opposite page.*)

ment of the central cells with fibroblasts (Figs. 10 and 11). Elongation of the preameloblasts, formation of terminal bars, and Tomes processes also occurred (Fig. 11). The stratum intermedium differentiated into cuboidal epithelium. Changes in the papilla were equivalent to that observed at eight days of cultivation. As observed in the twelve-day specimen, the enamel organ continued to develop and the papilla, though viable, failed to advance.

Fig. 12 illustrates the development at twenty days of cultivation. The specimen was flat and the sections showed a normal attempt toward cusp formation. The morphologic development is equivalent to that observed one day after birth at which time the entire crown is outlined. The outer tunic presented epithelial rests between which fibroblasts of the follicle penetrated to occupy the central-cell area of the enamel organ (Fig. 12). Epithelial pearl formation had also occurred in the area of the outer tunic. The inner cells of the stratum intermedium had differentiated into the cuboidal form (Fig. 13), and the inner enamel epithelium elongated particularly in the areas lateral to the cusp tip at the dentinoenamel junction (Fig. 13). Differentiation of the peripheral cells of the papilla was retarded, showing no more development than the eight-day cultures (Fig. 13). The developmental relation of ameloblast and odontoblast as observed in vivo[12] had failed to occur.

DISCUSSION

The study of "on the clot" cultivation of tooth germs presents another method of culturing the explant, using the Carrel flask technic. In evaluating the results, two controls were used. The results of "on the clot" cultivation[9] provided a means of comparison for the two methods, and the odontogenesis of the rat molar provided the chronologic standards used to evaluate development.[12]

Observations of growth and development are possible only when the explant is translucent. Both "in" and "on the clot" cultures are opaque upon planting and within four days become sufficiently translucent to exhibit the tooth germ elements. In "in the clot" cultivation, the translucency persisted for the twenty-day period of cultivation. When cultivated "on the clot," the explant was translucent from the fourth to the sixteenth day at which time the tooth germ became opaque and remained so until the specimens were taken at twenty days (Figs. 1 and 2). There is no present indication of the cause for the opacity found at sixteen days in "on the clot" cultivation.

Rapid growth and development occurred in "in the clot" cultivation using both chick and rat embryo extract[2, 9]; and also, in this investigation, this finding suggests that, at the time of planting, the cells possessed a residual growth potential unquestionably provided by optimum nutritional media and environment in vivo, and that, after planting, the environment and media are inadequate.

Gross observations of the living tooth germ offer a means of recording growth and development. From subjective and photographic records, it

appeared obvious that growth occurred until the sixteenth day of cultivation (Figs. 1 and 2). This finding was verified by histologic observation of dividing cells in the proliferative zone, which is a manifestation of the normal growth pattern of rat molar tooth germs.[12] The apparent decrease in size found in the latter stages of cultivation cannot be explained. It is interesting to point out that even though shrinkage did occur, there was no degeneration present in the tooth germs after twenty days of cultivation.

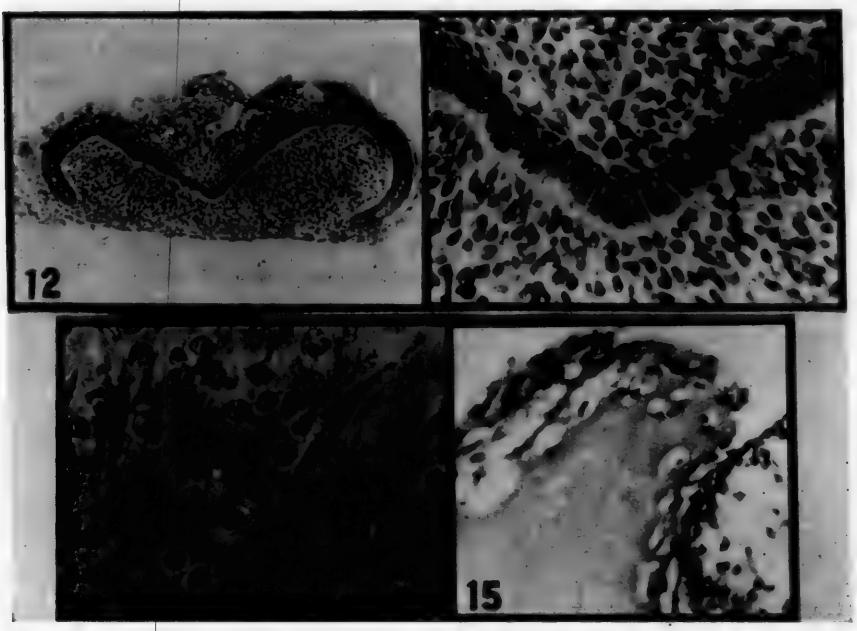


Fig. 12.—Nineteen-day rat embryo molar tooth germ cultivated 20 days. Normal morphology. Outer tunic shows epithelial remnants and pearl formation. Central-cell area is occupied by fibroblasts. Preameloblasts are elongated. No odontoblasts are present. (Orig. mag. ×150; reduced ⅔.)

Fig. 13.—High magnification of Fig. 12. Arrow points to differentiated cells of stratum intermedium. Polarity of preameloblasts reversed. No odontoblasts in papilla. (Orig. mag. ×450; reduced ⅔.)

Fig. 14.—Monocytes and fibroblasts in outgrowth. Twelve days' cultivation. Photographed in flask. (Orig. mag. ×430; reduced ⅔.)

Fig. 15.—Section of coagulum with monocytes. Note liquefaction of plasma and monocytes in situ. (Orig. mag. ×450; reduced ⅔.)

Observations of development were possible during the twelve-day period of translucency, depending on the orientation of the explant in the coagulum. The results were best when the tooth germ was planted on its buccal or lingual surface (Fig. 3). Unfortunately, it is impossible to orient the explant through the neck of the flask. A variation in equipment, now under consideration, may facilitate observations of development. Specimens fortuitously oriented made it possible to observe changes in the tooth germ elements, the most dramatic being the outline of the dentinoenamel junction, which is a yardstick in development of the explant.[13]

"On the clot" cultivation exhibited a marked difference in the survival of the outgrowth. The outgrowth in "in the clot" cultivation survived for the entire period of the experiment.[9] In the present study, the fibroblasts of the outgrowth continued to multiply for approximately twelve days (Figs. 1 and 2). At this time, there appeared in the area of the outgrowth numerous monocyte-like cells which seemed to have migratory potentialities (Figs. 1, 2, 14, and 15). The monocyte-like cells seemed to originate from the follicle and migrated to the periphery of the outgrowth. Coincidental with their appearance, large areas of liquefaction appeared,[1, 2] and the monocyte-like cells concentrated at the periphery of the digested clot. The process was progressive, and between sixteen and twenty days almost the entire outgrowth and coagulum surrounding the tooth germ was liquefied. The transition of fibroblasts into macrophages has been reported.[13] It is significant that only the outgrowth and not the original explant was lysed. It was impossible to determine whether the cells of the outgrowth had undergone senescense and death prior to the appearance of the monocyte-like cells. If this were so, it is likely that differentiation or formation of monocyte-like cells may have been associated with a chemotactic activity. The observation indicates that the fibroblasts of the outgrowth were viable at the time the monocyte-like cells appeared in the area (Figs. 2, *C* and 14). The reappearance of fibroblastic outgrowth at twenty days of cultivation suggests continuing this phase of investigation for longer periods of time.

A previous observation that the follicular wall preserves the gross morphology of the explanted tooth germ[9] is not substantiated by this study. The follicular wall was left intact in all cases and the majority of explants became flat after four days in the flask. This morphologic alteration did not seem to impair growth and development.

Previous investigation has established that rat embryo extract is a desirable nutrient medium if measured by survival.[9] The use of chick embryo extract resulted in degeneration of the papilla and odontoblasts after the first few days of cultivation,[2, 9] and the ectodermic elements showed greater survival potential than the mesodermic.[9] In previous and present studies, all explants survived the arbitrarily chosen period of twenty days. Losee[5] reported on seventy-eight days of cultivation of tooth germ in which the tissues remained viable. Szabō's[8] findings on survival are at variance with other investigators' results. However, the materials and methods used in his investigations were not similar to those of other workers in the field. At present, using rat embryo extract, survival presents no major problem.

The relationship between survival and development requires further examination. In this experiment, the pattern of development was not harmonious with survival. Development, like growth, was most rapid in the first period up to twelve days of cultivation. Considering the residual nutritional media present at the time of planting, the concept that dilute media is conducive to development requires further examination.

The results of "on the clot" cultivation as reported herein cast doubt upon the organizer relationship of the inner enamel epithelium and the odontoblastic layer. It is generally accepted that the enamel organ, and specifically the ameloblasts, exert an organizer influence on the peripheral fibroblasts of the papilla causing their differentiation into odontoblasts. The formation of the first layer of dentin is also presumed to influence amelogenesis.[16] These opinions have been supported by observations in tissue culture and transplantation studies.[2, 6, 14, 15] In a recent report on the odontogenesis of the rat molar, it was established that even though odontoblast and dentin formation occurs at the cusp tip, differentiation of the stratum intermedium does not occur, and no enamel forms in this area.[12] The present study casts further doubt on the concept that the presence of dentin is essential to functional differentiation of the ameloblasts. The present study also weakens the concept of organizer influence of the inner enamel epithelium on the odontoblasts. In the present study, the development of ectodermic and mesodermic elements of the tooth germ was inharmonious. There was unquestionably greater development of the inner enamel epithelium than of the peripheral fibroblasts of the papilla (Figs. 7, 9, 11, and 13). While in most cases development of the ameloblasts proceeded to a postnatal stage, the development of odontoblasts remained in a prenatal stage. Elongation of the ameloblasts, followed by formation of terminal bars and Tomes processes, progressed without differentiation of odontoblasts and the formation of predentin in the same area. Attempts to understand the lack of harmony in development and the failure to follow the in vivo pattern may be attributed to nutritional abnormality and the need for its diffusion into the deeper parts of the explant. On the other hand, the nutritional needs of both tissues may be different or require a delicate balance. In vivo, epithelium undergoes the complete cycle of cytomorphosis in the presence of tissue fluid whereas mesodermic elements require a direct blood supply. Should this be the case, the failure of differentiation and apposition of mesodermic tissue may be attributed to nutritional deficiency. This observation of nonparallel development seriously impairs the concept that stages in the cytomorphosis of the ameloblasts are dependent upon the formation of dentin. As previously noted, the presence of dentin does not necessarily cause functional differentiation of the ameloblasts[12] and, under the conditions of this experiment, maturation of the ameloblasts occurred without differentiation of odontoblasts. The factor which remained constant in both studies is the differentiation of the stratum intermedium which further suggests its role as the organizer of the ameloblasts.

CONCLUSION

"On the clot" cultivation, using the same media as "in the clot" cultivation, shows an advance in development of the enamel organ. The inharmonious development of ameloblasts and odontoblasts casts doubt on the present concepts of organizer influence in odontogenesis. The more rapid growth and development observed in early stages of development may be attributed to

the retained nutritional media in the tissues at the time of planting. The epithelial elements of the tooth germ have a greater growth and development potential than the fibroblastic elements in vitro.

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DEVELOPMENT OF THE SQUAMOSO-MANDIBULAR ARTICULATION IN THE RAT

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INTRODUCTION

ALTHOUGH the squamoso-mandibular articulation of the rat (and the mouse) has been studied frequently in experimental investigations,[1, 7] no report exists on the early development of this joint. The purpose of the present investigation was to study the developmental changes in the squamoso-mandibular articulation in the rat from 16 days insemination age to 30 days after birth.

REVIEW OF THE LITERATURE

Collins, Becks, Simpson, and Evans[8] described briefly the changes in the mandibular joint in female rats from 5 days to 465 days after birth. They showed that from 5 to 20 days, the condylar process of the mandible is conical and consists of hyalin cartilage covered by a layer of fibrous connective tissue. The part of the condyle which joins the mandibular body shows a zone of erosion and osteogenesis. From 25 days on, the diameter of the cartilage increases but its height decreases and at 30 days only a thin layer of cartilage covers the bony condylar process. They observed that the calcification of condylar cartilage prior to its erosion occurred only at 30 days and that both the cartilage growth and replacement by bone are progressively slowed down. Cabrini and Erausquin[9] described the anatomy, histology, and physiology of the squamoso-mandibular articulation in 5- and 6-month-old rats. According to these investigators the articular surface of the zygomatic process is covered by chondroid cells which are progressively transformed into osteoblasts and osteocytes. They showed that the condylar cartilage consists of zones similar to those seen in epiphyseal cartilages. The meniscus is formed by interwoven collagenous and elastic fibers and shows cells which resemble chondrocytes. The peripheral part of the disc contains adipose tissue. They observed further that due to resorption on its inferior border and apposition on its superior surface, the zygoma progressively shifts upward.

Levy,[10] studied the growth of the mandibular joint in normal mice ranging in age from birth to 540 days. He stated that the interarticular disc is composed of loose connective tissue at birth but with age it becomes less cellular and denser. He described the manner in which the condylar cartilage is re-

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placed by bone and showed that the rate of cartilage growth and replacement decreased with age but that the cartilage maintained its growth potential even in the oldest animal.

Weinmann[6, 7] briefly described the condylar process of the normal young rat and reported the changes in this structure in rickets and hyperparathyroidism. Reference to the prenatal differentiation of the squamoso-mandibular articulation of the rat was made by Bhaskar[11] who, in discussing the development of the rat mandible, mentioned the first appearance of the synovial cavities and early formation of the articular disc.

MATERIAL AND METHODS

The present study is based on a histologic investigation of complete serial sections through the squamoso-mandibular joint of 31 normal and 30 *ia* rats ranging from 16 days insemination age to 30 days after birth (Table I). The *ia* is a mutant strain[12] characterized by an absence or marked retardation of physiologic resorption of bone.[13, 14] This characteristic of the *ia* skeleton leads to the accumulation of successive layers of bone formation and the persistence of perichondral bony splints around cartilages which undergo endochondral ossification. Thus, the growth pattern of bones and cartilages can be traced.[11, 14, 15] The developmental changes in the fossa, articular disc, capsular ligament, synovial cavities, and the articular part of the condylar process were studied in *ia* and in normal littermates.

TABLE I
AGE AND NUMBER OF ANIMALS IN WHICH THE SQUAMOSO-MANDIBULAR ARTICULATION WAS STUDIED IN SERIAL FRONTAL SECTIONS

AGE	*ia* ANIMAL	NORMAL LITTERMATES
16 days insemination age to birth	10	13
1-4 days	8	3
5-10 days	7	7
12-30 days	5	8
Total	30	31

After sacrifice, the heads of these animals were fixed in 5 per cent formalin, decalcified in 5 per cent nitric acid, embedded in celloidin, cut serially in a frontal plane, and stained with hematoxylin and eosin.

The age of the specimens was determined as follows: (1) For animals obtained from pregnant females before birth, insemination age was calculated from the night of mating to the day of recovery of the fetus. (2) For animals from birth to 5 days after birth, the time of parturition was observed within a range of fifteen to thirty minutes. (3) For animals older than 5 days, age calculations were made from the forenoon or afternoon of parturition.

FINDINGS

Differentiation of the Squamoso-mandibular Joint.—The rat mandible begins to ossify at 16 days insemination age by the formation of a bony plate lateral to Meckel's cartilage. At 17 days insemination age, the future molar region

of the mandible consists of 2 lateral and 2 medial plates of bone which enclose the alveolar trough. At this time, a sheet of cartilage differentiates between the 2 lateral bony plates. From this cartilaginous sheet, a superior and an inferior focus of cells proliferates posteriorly as the condylar and angular cartilages, respectively (Fig. 1). As these rod-shaped cartilaginous masses extend posteriorly, they diverge from each other first in the sagittal and later also in the horizontal plane.

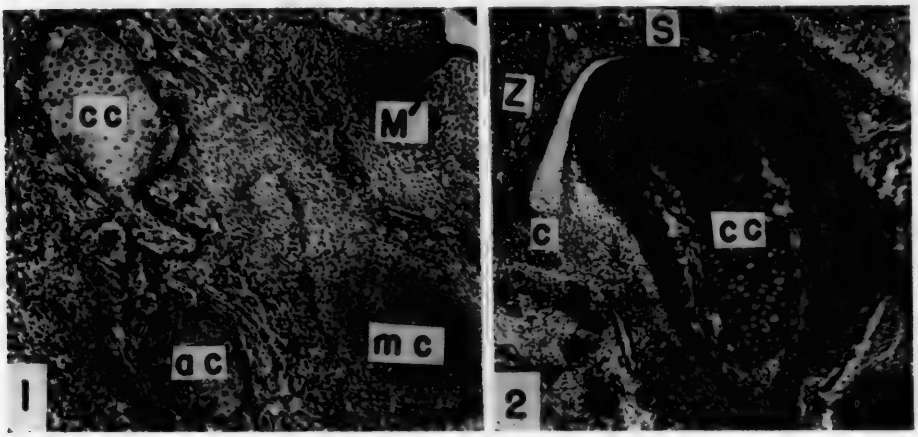

Fig. 1.—Frontal section through the mandibular ramus at 17 days insemination age. The condylar cartilage (*CC*) is enclosed in the bone. *AC*, angular cartilage; *MC*, Meckels cartilage; *M*, molar tooth germ. (Orig. mag. ×85; reduced ⅜.)

Fig. 2.—Section through the squamoso-mandibular joint at 19 days insemination age. Note the appearance of the first slit, *C*. *S*, squamosal bone; *Z*, zygomatic process of the squamosal. (Orig. mag. ×85; reduced ⅜.)

At this stage, the posterior end of the condylar cartilage consists mainly of mesenchymal cells and prechondrocytes, and lies lateral to the future alisphenoid. Between 18 and 19 days insemination age, ossification of the squamosal bone begins with the formation of a bony plate, the squama (Fig. 2). Its lower border overlaps the lateral border of the alisphenoid and extends from here upward toward the parietal or lateral side of the head. From the squama, the zygomatic process projects outward and forward. The posterior end of the condylar cartilage lies inferomedially and in close proximity to the zygomatic process from which it is separated by highly cellular mesenchyme. Within this mesenchyme, at 19 days insemination age, a slit appears superolaterally to the condylar cartilage (Fig. 2). At 20 days insemination age, this slit extends into the mesenchyme superiorly to and then superomedially to the condyle (Fig. 3). Also, at this stage, another slit appears in the mesenchyme between the first slit and the superolateral surface of the condylar cartilage (Fig. 3). These slits are bordered by flattened fusiform mesenchymal cells which are oriented with their long axes parallel to the surface of the slits. The superior and the inferior slits are separated by a layer of mesenchymal cells which is the anlage of the articular disc.

In the lateral part of the articulation, fusiform mesenchymal cells can be seen extending from the inferior border of the zygomatic process to the perichondrium of the condylar cartilage. These cells are the anlage of the capsular ligament.

Thus, at 20 days insemination age, the squamoso-mandibular articulation differentiates. The articular fossa of the joint is formed by the inferomedial surface of the zygomatic process and the inferolateral surface of the lower part of the squama. Anteroposteriorly, the fossa resembles a channel while, mediolaterally, it is deeply concave. The condylar cartilage which is convex superiorly lies within the concavity and is covered by a thick perichondrium. The latter is separated from the periosteum covering the articular surface of the fossa by 2 slits which are in turn separated by the anlage of the articular disc. The lateral boundary of the articulation is formed by the anlage of the capsular ligament while medial to the condylar cartilage and separating it from the alisphenoid, the bundles of the external pterygoid muscle can be seen.

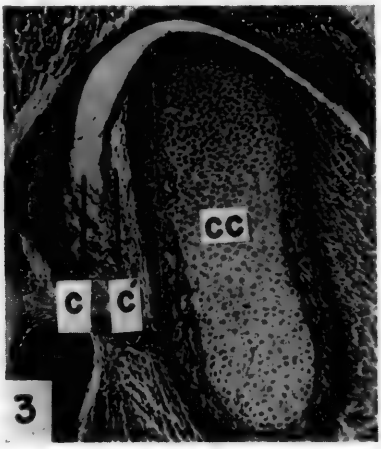

Fig. 3.—At 20 days insemination age. Note the appearance of the second slit, *C'*. (Orig. mag. ×70; reduced 3/8.)

Changes in the Disc.—The anlage of the disc, which arches over the condyle between the slits, at first consists of fusiform cells arranged parallel to the articular surfaces (Fig. 4) and showing many mitotic figures. The disc is wide in its lateral part where it is 5 to 6 cell layers thick, then narrows and becomes indistinct medially. By 21 days insemination age, it can be seen to be continuous laterally with the anlage of the capsular ligament and medially with the connective tissue around the fibers of the external pterygoid muscle (Fig. 3).

At the second day after birth, a few collagenous fiber bundles, running primarily in an anteroposterior direction, are distinctly seen in the disc. By the fourth day, the bundles increase in number. Although the majority of them can be seen running in an anteroposterior direction, other fibers also run

mediolaterally in the anterior and posterior parts of the disc. On the fifth day, the width of the disc in the anterior and posterior parts of the fossa increases considerably but it remains narrow centrally. From five to thirty days after birth, the number of collagenous fiber bundles increases progressively and the disc becomes a highly fibrous structure with relatively few cells (Fig. 5). But while the number of fibers increases with age, their general orientation in the disc remains essentially the same, i.e., the anteroposterior fibers run through the entire length of the disc and are especially concentrated in its medial part. The mediolateral fibers are present in large numbers only in the anterior and posterior portions of the lateral two-thirds of the disc.

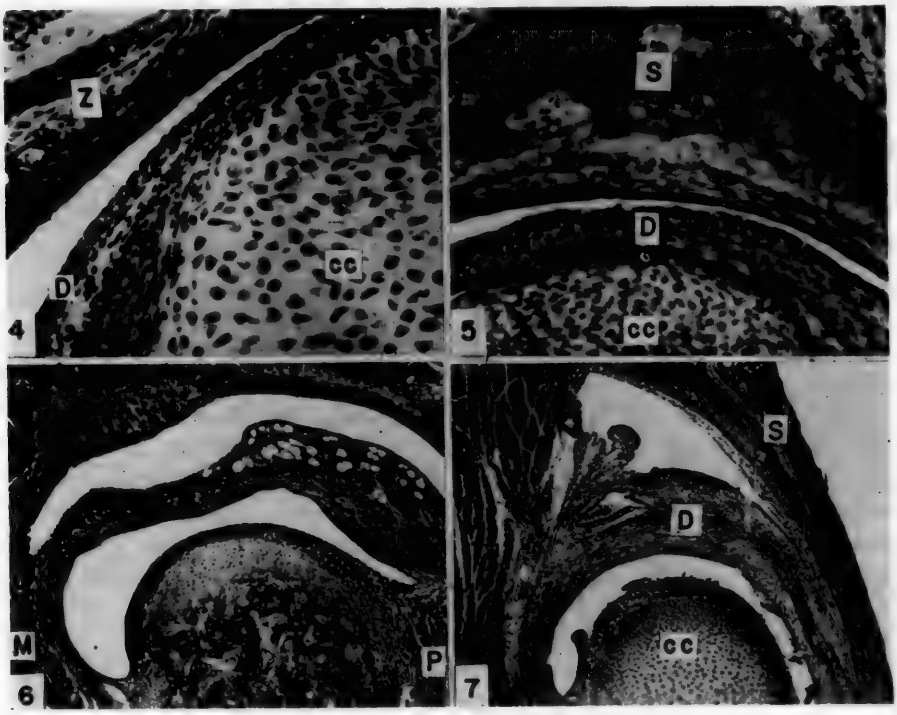

Fig. 4.—At 21 days insemination age. Note the highly cellular disc, *D*, *CC*, condylar cartilage; *Z*, zygomatic process. (Orig. mag. ×310; reduced ⅓.)

Fig. 5.—Twenty days after birth. Note the collagenous nature of the disc. The fibers are mainly cut in cross section. Also note the rapid bone apposition in the depth of the fossa. *S*, squamosal. (Orig. mag. ×310; reduced ⅓.)

Fig. 6.—Twenty-five days after birth. Photomicrograph of a frontal section through the anterior part of the synovial cavities. *M*, masseter muscle; *P*, external pterygoid.

Fig. 7.—Twenty-three days after birth. Photomicrograph of a section through the posterior part of the synovial cavities. (Orig. mag. ×85; reduced ⅓.)

Anterior to the area of the articulation, the disc thickens (Fig. 6) and its fibers become continuous with the epimysium of the external pterygoid muscle, medially, and with the connective tissue surrounding the masseter muscle, laterally. The part of the disc posterior to the articulation is also thicker than

in the center (Fig. 7). Medially in this area, its fibers are continuous with the epimysium of the external pterygoid muscle and laterally with the capsular ligament and the connective tissue surrounding the temporal muscle.

Between 17 and 19 days after birth, blood vessels and a pad of fat appear in the anterior part of the disc (Fig. 6). At 23 days, a similar fat pad and small blood vessels are seen in the posterior portion of the disc.

Changes in the Articular Cavities.—By 21 days insemination age, the slits increase in size and extend from the superolateral to the superior aspect of the condylar cartilage. Their medial terminations are obscured by the proximity of the condyle to the articular surface of the squamosal bone. The contour of the synovial cavities follows the general outline of the articular fossa. However, in the posterior part of the articulation, the cavities extend farther laterally than the fossa. By the fourth day after birth, the slits or the synovial cavities are better defined in the anterior and posterior parts of the articular fossa than centrally. The superior cavity is the larger and its medial extremity extends for some distance between the external pterygoid muscle and the outer plate of the squama. The medial extremity of the inferior slit ends superomedially to the condylar cartilage.

In the central part of the articulation, the lining of the synovial cavities is formed by a single row of flattened fusiform cells, the long axes of which are parallel to the cavities. However, in the anterior and posterior parts of the cavities, a layer of short cuboidal cells which is 3 to 4 cells thick forms the lining (Fig. 6).

At 15 days after birth, in the posterior part of the squamoso-mandibular articulation, a villus-like growth extends from the capsular connective tissue into the superior synovial cavity (Fig. 7). This villus becomes larger and more pronounced with age. At 23 days, it is covered by 2 or 3 layers of flattened cells and its core is formed by well-vascularized connective tissue. At 23 days, a similar villus extends into the lateral part of the inferior slit from the connective tissue over the superolateral aspect of the condylar cartilage (Fig. 7). However, it is much smaller than the one related to the superior slit and its mesenchymal core is not as well vascularized. By 30 days, a number of villi can be seen projecting into the posterior parts of the superior and inferior slits. These villi are largest in the superior synovial cavity and there they extend mainly from the lateral wall of the cavity.

Changes in the Articular Fossa.—At 21 days insemination age, the squama as well as the zygomatic process consist of 2 bony plates which enclose areas of hemopoietic marrow (Fig. 8). The fossa is formed by the inferior surface of the zygomatic process and a portion of the squama (Fig. 8).

At 20 and 21 days insemination age, the surface of the fossa shows bony apposition except on the articular surface of the squama, medial to the condyle, where resorption occurs. At this age, the most rapid apposition occurs at the inferior border of the zygomatic process (Fig. 9). This area of rapid growth

leads to the progressive deepening of the fossa. The anteroposterior lengthening of the articular fossa occurs by progressive apposition at the anterior margin of the root of the zygomatic process.

Between 1 and 2 days after birth, the apposition and resorption pattern in the articular fossa changes slightly. The portions of the articular fossa which face the superolateral and superomedial surfaces of the condylar cartilage show resorption lacunae, while the part of the fossa facing the superior surface of the condylar cartilage, and the inferior border of the zygomatic process show rapid apposition of bone (Fig. 9). The appositional growth of the inferior

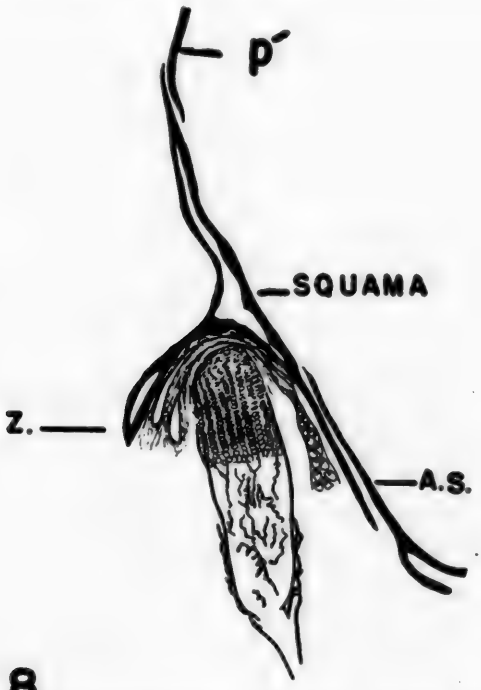

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Fig. 8.—Diagram of the squamoso-mandibular articulation showing the relationship between the squamosal, the parietal (p'), and the alisphenoid, *A.S.* *Z*, zygomatic process.

border of the zygomatic process is in an inferolateral direction. These areas of rapid bone formation are characterized by the presence of wide layers of lightly staining osteoid. The latter is particularly rich in cells and only superficially resembles cartilage. Although the rate of bone apposition and resorption varies from time to time, the pattern of bone formation and destruction in the fossa remains unchanged until 30 days after birth (Fig. 10). This pattern of growth in the fossa leads to its progressive widening. However, the apposition in the depth of the fossa keeps pace with the growth at the inferior border of the zygomatic process. This prevents an excessive deepening of

the fossa. Up to 30 days after birth, the temporal surface of the zygomatic process continues to show bone apposition. To compensate for the resorption on the articular surface of the squama, its endosteal surface shows apposition of bone.

At 4 days after birth, a part of the masseter muscle can be seen to originate from the lateral surface of the most anterior part of the fossa (Fig. 11). With age, however, the area of the origin of the muscle extends anteroposteriorly so that at 30 days it originates from the lateral surface of the anterior half of the fossa (Fig. 10). From their origin, the muscle fibers run downward and inward and lie laterally to the condylar cartilage where they function as a capsular ligament.

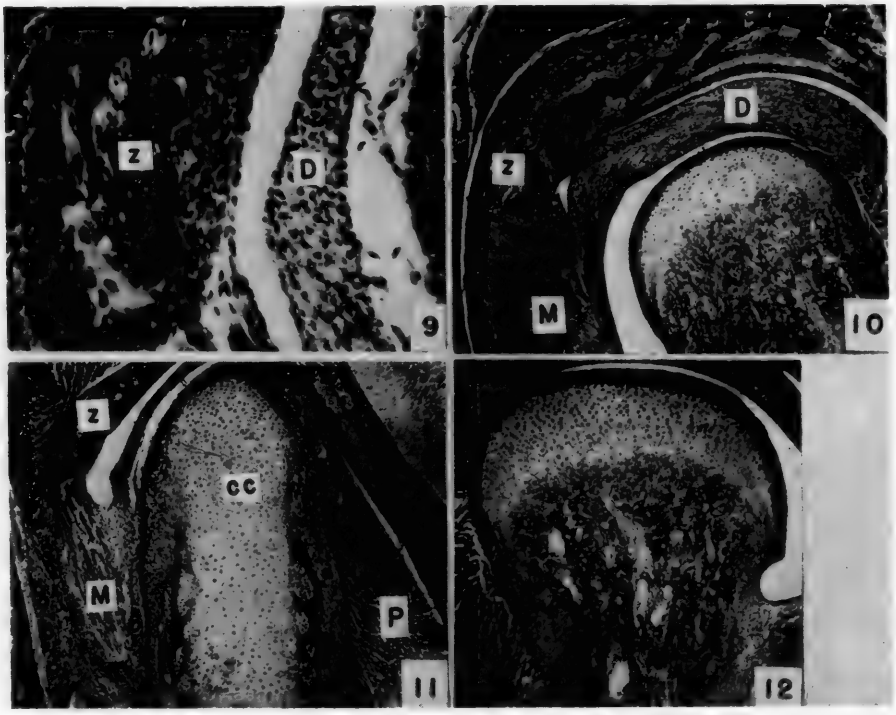

Fig. 9.—Two days after birth. Resorption is seen on the articular fossa superolaterally to the condylar cartilage while apposition appears on the inferior border of the zygoma, *Z*. (Orig. mag. ×310; reduced ⅓.)

Fig. 10.—Twenty-one days. Resorption occurs on the articular fossa superolaterally to the condylar cartilage. Apposition is seen in the depth of the fossa. (Orig. mag. ×77; reduced ⅓.)

Fig. 11.—Four days after birth. Note the relationship of masseter muscle (*M*) to the zygomatic process and the bone apposition on the sides of the condylar process. *P*, external pterygoid. (Orig. mag. ×77; reduced ⅓.)

Fig. 12.—Thirty days after birth. Note the modeling resorption on both the medial and lateral sides of the condylar neck. (Orig. mag. ×77; reduced ⅓.)

The syndesmosis between the lower part of the squama and the upper part of the alisphenoid is a site of active proliferation. The connective tissue cells are oriented in the typical three zones seen in all growing sutures and the

opposing surfaces of the bones are bordered by osteoid zones. This growth leads to an upward and outward shift of the squamosal bone and a progressive increase in the distance between the articular fossa and the base of the skull.

Changes in the Condyle.—At 17 days insemination age, the condylar cartilage is enclosed, except for its most posterior end, in the body and ramus of the mandible (Fig. 1). The posterior portion of the cartilage is surrounded by a large mass of mesenchymal cells and prechondrocytes. The perichondral bony splint surrounds the cartilage everywhere except at its posterior end. At this stage, the growth of the condylar cartilage is primarily in a posterior direction. At 18 days insemination age, the superolateral surface of the condylar cartilage as well as the posterior surface shows a zone of appositional growth. Up until 18 days insemination age, the condylar cartilage which extends from the first molar region of the mandibular body to an area beyond the ramus, is a solid rod of cartilage. Its chondrocytes in the molar region are the oldest while those at the posterior end belong to the youngest generation. At 19 days insemination age, a zone of cartilaginous destruction appears in the region of the first molar tooth germ. Here, the chondrocytes first become edematous and their scanty intercellular matrix becomes deeply basophilic. Simultaneously, the mesenchymal cells invade the perichondral splint and later destroy the cartilaginous capsules. Soon the cartilage cells disappear and the partly destroyed perichondral splint surrounds young connective tissue in which macrophages and some giant cells can be recognized. At 20 days insemination age, the zone of destruction extends into the ramus to a point just posterior to the coronoid process. By 21 days insemination age, this destruction has reached the cartilage in the posterior part of the ramus.

Throughout this time while the main path of growth of the cartilage is posteriorly, some growth in a superolateral direction does occur. Between the third and fourth day after birth, the superolateral component of condylar growth increases considerably. This is evidenced by the fact that the proliferating zone of mesodermal cells on the superolateral aspect of the condyle greatly increases in thickness, and that any given frontal section through the condylar cartilage which previously showed chondrocytes of the same age now reveals cells of different generations. There is a layer of closely packed mesodermal cells beneath the inferior slit of the squamoso-mandibular articulation. These cells overlie the superior and superolateral aspects of the condyle. Beneath this layer is a transition zone consisting of prechondrocytes and chondrocytes. Inferior to these cells, an area exists in which the chondrocytes become swollen and edematous and are separated from one another by a hyalin intercellular matrix. Below this area is another zone in which the hyalin matrix is calcified.

The condylar cartilage continues to grow primarily in a superolateral direction until the nineteenth day of life. At this time, the main mass of the proliferating zone shifts slightly medially, resulting in a more superior path of growth. This continues to be the chief direction of proliferation through the thirtieth day.

At 4 days, no cartilage destruction is evident in central sections through the articulation but by the sixth day it has extended into this area. At this time the cartilage from the zone of destruction through the prechondrocyte layer is approximately 55 cells thick. The destruction continues to encroach upon the condylar cartilage at a greater rate than new cartilage is proliferating. Therefore, the thickness of the cartilaginous cap of the condyle gradually decreases, until at 30 days, it is only 10 to 15 cells thick. At this time, it is more or less crescent-shaped and surmounts the condylar process (Fig. 12).

In the beginning, the condylar process consists almost entirely of cartilage. As age advances, however, it consists of an increasing amount of bone which is surmounted by cartilage. At 20 days insemination age, the process is club-shaped and its medial and lateral bony surfaces are approximately parallel to each other. Apposition of bone occurs on both surfaces until the fifteenth day after birth (Fig. 11). Between 15 and 19 days after birth, modeling resorption begins on the medial and lateral walls and becomes quite marked at 23 days after birth (Fig. 12). As a result of this pattern of resorption, at 30 days the condylar process consists of a narrow bony neck which is surmounted by a mushroom-shaped condylar cartilage.

Changes in the Capsule.—At 19 days insemination age and coincident with the appearance of the first slit of the articulation, a condensation of mesenchyme is seen extending between the zygomatic process and the lateral aspect of the posterior end of the condylar cartilage. At birth, this condensation becomes a band of flattened fusiform cells with a few collagenous fiber bundles. It is continuous above with the periosteum covering the inferior border of the zygomatic process, and extends inferomedially to become continuous with the mesodermal cells surrounding the posterior end of the condylar cartilage. Throughout the articulation, the lateral side of the interarticular disc unites with the capsule approximately halfway between its superior and inferior attachments. The most posterior part of the capsule is poorly defined at birth. At the fourth day, the posterior extremity of the capsule and the disc can be seen to taper and blend with the periosteum covering the squamosal bone behind the root of the zygoma.

After 4 days, a portion of the masseter muscle originates from the anterior part of the lateral wall of the articular fossa and with age the extent of this area increases (Figs. 10 and 11). Thus, the capsular ligament can be identified only in that part of the articulation where the masseter muscle does not lie laterally to the condylar cartilage.

By 15 days, the capsule has greatly increased in width but it is still a highly cellular structure. At this time a small fold extends from the capsule into the superior synovial slit. Similar villi project into the inferior slit from the capsular wall. These are smaller and less numerous than those related to the superior slit. From 15 to 30 days, the capsule can be seen only in the posterior half of the articulation where it extends from the

inferior border of the zygomatic process to the condylar process immediately below the condylar cartilage and consists of a progressively increasing number of collagen bundles.

The medial side of the articulation is not limited by a capsule as is its lateral side. Here, the external pterygoid muscle performs the function of the capsular ligament.

DISCUSSION

Nomenclature.—The articulation between the rat (and mouse) mandible and the skull has been referred to as the mandibular joint[1, 2, 3, 5, 8, 10] or as the temporo-maxillary joint.[9] Since the temporal bone does not exist in the rat or the mouse and since the mandibular condyle in these animals articulates with the zygomatic process and the squama of the squamosal bone, this joint should be properly referred to as the squamoso-mandibular articulation.

Direction of Growth of the Condylar Cartilage.—From 17 days insemination age to 4 days after birth, the main direction of growth of the condylar cartilage is backward. This contributes primarily to the linear growth of the mandible. Starting at four days, however, the condylar cartilage starts to grow upward. This upward component of condylar growth increases with age and leads to a downward shift of the mandible. These different directions of growth at the condylar cartilage are closely related to the developmental changes in the dental lamina and the tooth buds. It is known that the dental lamina forms the first, second, and third molar tooth germs at 14 to 15 days insemination age, 19 days insemination age, and 10 days after birth, respectively.[11] The prenatal and early postnatal phase of dental development, therefore, consists of an anteroposterior growth, first of the dental lamina and then of the first and second molar tooth germs. This phase of dental development coincides with the phase of condylar growth which leads primarily to an anteroposterior lengthening of the mandible.

Early in postnatal life, the molar tooth germs begin to grow in height; their hard tissues begin to form and their prefunctional eruptive movement starts. Coinciding with this phase of dental growth, the condylar cartilage grows in an upward direction and thus permits the vertical growth of the alveolar process.

It has been demonstrated in this study that one area of appositional growth at the condylar cartilage is on its superolateral surface. This site of growth in the right and left condylar cartilages leads to a progressive increase in the distance between the cartilages. Since the formation of bone follows the cartilaginous growth, this pattern of condylar growth serves to widen the posterior ends of the mandible and thus keep pace with the widening cranial base.

Replacement of the Condylar Cartilage by Bone.—The replacement of the condylar cartilage by bone begins at 19 days insemination age. It is well known that in endochondral ossification, prior to its destruction, the cartilage matrix calcifies.[16] This, in routine histologic sections, is seen as a deeply basophilic zone. Although in the present investigation a definite change in the cartilage

matrix could not be clearly seen at 19 days insemination age, at 21 days insemination age the calcified zone of the cartilage could be demonstrated. This finding is in disagreement with that of Collins, Becks, Simpson, and Evans,[8] who reported that the calcification of the condylar cartilage does not appear until 30 days after birth.

The Articular Disc.—The articular disc originates from the mesenchyme between the superior and inferior slits and up to birth consists predominantly of mesenchymal cells. With age, the most striking change in its structure consists of the appearance of collagen bundles which become progressively thicker and more numerous. Although in some areas these fibers run mediolaterally, the predominant direction of the fibers is anteroposteriorly. Since the main direction of movement of the squamoso-mandibular articulation in the rat is anteroposteriorly this arrangement of the fibers in the disc is not surprising.

The general shape of the articular disc is similar to that seen in other articulations. It is narrowest in the area of the actual articulation, while anteriorly and posteriorly it widens. Medially, the disc becomes continuous with the connective tissue surrounding the external pterygoid muscle. This relationship between the disc and the external pterygoid muscle would indicate that during the forward movement of the condyle the disc is also brought forward.

The presence of chondroid cells has been reported in the articular disc of 5- to 6-month-old rats.[9] In the present investigation, such cells could not be demonstrated. Cabrini and Erausquin[9] reported the location of fat and blood vessels in the anterior and posterior portions of the disc. The findings in the present investigation confirm this observation.

The Articular Fossa.—The articular fossa is formed by the zygomatic process and a portion of the squama of the squamosal bone. The predominant pattern of apposition and resorption in the fossa is as follows: on the inferior border of the zygomatic process which forms the lateral lip of the fossa, rapid apposition of bone occurs. The direction of growth here is inferolaterally and this pattern tends to widen as well as to deepen the fossa. However, the deepening of the fossa is prevented by rapid appositional growth in the depth of the fossa directly above the condylar cartilage.

Resorption of bone occurs on the parts of the articular fossa which face the superolateral and superomedial parts of the condylar cartilage. These sites of resorption lead to an increase in the transverse width of the fossa which goes hand in hand with the progressively enlarging condylar process.

Cabrini and Erausquin,[9] who reported the only detailed work on the squamoso-mandibular joint of the rat, described a "rising" of the zygomatic process. This upward shift was ascribed to resorption on the articular and apposition on the temporal surfaces of this process. In the present investigations, however, the marked upward shift of the articular fossa was found to be primarily due to very active growth in the squamoso-alisphenoidal suture. This site of growth led to a lengthening of the lower part of the squama and a progressive increase in the distance between the articular fossa and the base of the skull. The animals used by Cabrini and Erausquin[9] were 5 and 6

months old. It is conceivable that at that age resorption and apposition of bone play a dominant role in the upward shift of the zygomatic process and the fossa.

Chondroid cells or cartilage-like tissue have been described in the depth of the fossa[8, 9] and it has been stated that these cells progressively change to osteoblasts and osteocytes. In the present investigation, this area of the fossa showed rapid bone apposition. This was evidenced by a wide zone of pale osteoid tissue with many large osteocytes. A similar type of tissue was seen at the inferior border of the zygomatic process. This tissue is not cartilage but uncalcified immature bone. This assumption is based on the fact that the intercellular substance of this tissue does not stain like cartilage and secondly, when this tissue calcifies, it stains like bone rather than calcified cartilage.

The Capsular Ligament.—In the squamoso-mandibular joint of the rat, the capsular ligament is not well defined. On the medial side of the joint, it does not exist, while on the lateral side, up to 4 days of age, it extends from the entire length of the lateral boundary of the fossa to the neck of the condylar process. At 4 days, the anterior part of the lateral boundary gives origin to a portion of the masseter muscle, but this area later extends posteriorly. With increasing age, the capsular ligament can be identified only in the posterior half of the lateral surface of the articulation where it is well defined. The absence of a distinct capsular ligament on the medial and anterior half of the lateral surface of the zygomatic process is probably due to the presence of the external pterygoid and the masseter muscles in these areas. These muscles, especially the masseter, perform the function of a capsular ligament.

SUMMARY

This histologic investigation is based on complete serial frontal sections through the squamoso-mandibular articulation of 31 normal and 30 *ia* rats ranging from 16 days insemination age to 30 days after birth. The *ia* rats are a mutation in which bone resorption does not occur and the pattern of bone and cartilage formation can be accurately traced.

This study showed that the condylar cartilage differentiates in the body of the mandible at 17 days insemination age and grows backward toward the zygomatic process of the squamosal bone. In the mesenchyme which separates the cartilage from the bone, a superior and an inferior slit appear at 19 and 20 days insemination age respectively. At 21 days insemination age, the anlage of the capsular ligament appears and thus at this age the squamoso-mandibular articulation differentiates.

In the articular disc which at this time consists only of fusiform cells, some collagen fibers appear at 2 days after birth and their number progressively increases with age. Their general orientation is anterior-posterior. In the central part of the articulation, the disc is narrowest while medially, laterally, and especially, anteriorly and posteriorly, it widens. In the latter two areas, it contains blood vessels and fat cells but elsewhere it is avascular.

The synovial cavities are lined partly by flattened and partly by cuboidal epithelium. At 15 days after birth, villi begin to form and extend into the anterior and posterior portions of the synovial cavities. These villi have a vascularized core and their number and size increases with age. The articular fossa is formed by the zygomatic process and the inferior half of the squama of the squamosal bone. Generally rapid bone apposition occurs on the inferior border of the zygomatic process and the depth of the fossa and resorption occurs on the surfaces of the fossa which are superolateral and superomedial to the condylar cartilage. Thus the fossa widens while its depth remains more or less constant. The direction of the growth of the condylar cartilage is first predominantly backward but later it shifts to a more upward and outward direction. These directions of growth are related to the development and growth of teeth. The destruction of condylar cartilage and its replacement by bone begins at 19 days insemination age and progressively reduces the cartilaginous part of the condylar process. Modeling resorption of the neck of the condylar process begins at 15 days after birth. The capsular ligament of the squamoso-mandibular articulation of the rat is not well marked. It extends from the posterior half of the lateral rim of the fossa to the condylar neck. In the anterior part of the lateral surface of the articulation, the masseter muscle and medially the external pterygoid act as the capsule.

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THE HISTOCHEMISTRY OF THE ADRENAL CORTEX FOLLOWING REMOVAL OF THE MAJOR SALIVARY GLANDS

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PREVIOUS experiments in this laboratory have shown that removal of the major salivary glands was accompanied by uterine and testicular weight alterations[1, 2] and also histologic changes in the adrenal cortex.[1, 3] In order to provide further evidence as to the nature of these adrenal changes a histochemical analysis of the cortex was performed utilizing the methods described by Dempsey and Wislocki[4] and others.[5, 6] A histologic examination of the ovaries and uteri of the desalivated animals was also performed.

PROCEDURE

A total of 70 weanling rats of the Sprague-Dawley strain were used in these studies. The animals were divided into 3 experimental groups; one group was desalivated by a procedure already reported,[2] another was sham-operated, and a third group was unoperated and served as a control. After a 48-hour recovery period, the animals were divided into groups of 8 rats each, consisting of 2 male and female desalivates and one each of both sexes of sham-operated and control animals. At 3, 4, 6, 10, 16, 24, and 32 days following surgery, the animals were sacrificed and the uteri and ovaries removed and fixed in 10 per cent neutral formalin, embedded in paraffin, sectioned serially at 12μ, and stained with hematoxylin and eosin. The adrenal glands were removed and studied according to the following procedure. One gland from each animal was fixed in neutral formalin and sectioned at 15μ on a freezing microtome and one section was stained with Sudan Black B, one with Sudan IV, and another by the Schiff "plasmal" method. These sections were affixed to microscope slides with 1 per cent gelatin, hardened by formaldehyde vapors and subsequently mounted in glycerogel. Two additional sections were mounted unstained, one of which was extracted for 30 minutes in acetone and the other was untreated. These sections were compared under a polarizing microscope for acetone soluble droplets in order to demonstrate birefringence. This series of reactions characterizes the lipid droplets of steroid-secreting glands, and are of interest since variations in reactivity have been correlated with different degrees of functional activity.[5, 7]

One adrenal gland from every group was fixed in acidified silver nitrate for ascorbic acid demonstration. After fixation for one-half hour, the glands

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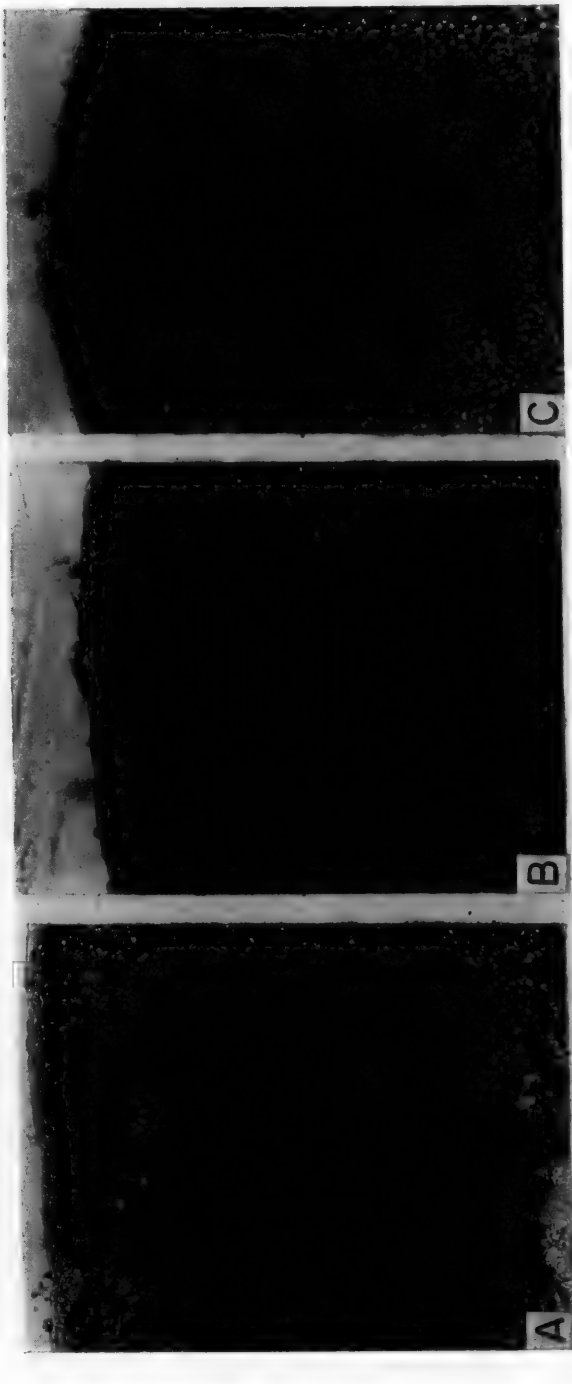

Fig. 1.—Frozen sections of formalin-fixed adrenals stained with Sudan Black B. (Orig. mag. ×200; reduced ⅔.) *A*, Control female; *B*, desalivated female, both 48 hours after surgery. A transitional zone is absent in desalivated animal. *C*, Illustrates the adrenal cortex of a desalivated female at the fourteenth day. The transitional zone has reappeared and is comparable to the control animals.

were transferred to an acidified sodium thiosulfate solution for one hour and washed in running water, embedded in paraffin, sectioned at 5μ, and counterstained with eosin. Another adrenal gland was embedded in paraffin and sectioned serially at 6μ. These sections were stained in hematoxylin and eosin and mounted in glycerogel.

RESULTS

Sudan IV and Sudan Black.—The control and sham-operated animals showed the following results throughout the entire experimental period: The glomerulosa and fasciculata stained intensely and the reticularis only moderately (Fig. 1, *A*). The lipid material was present characteristically as a mixture of equal numbers of coarse and fine droplets. This appears to indicate that the effect of the sham operation itself was not sufficient to dramatically alter these tissues. However, pronounced differences were noted in the adrenal gland of the desalivated animals (Fig. 1, *B*). During the first six experimental days the intensity of the Sudan stains in the zona fasciculata was markedly increased as compared to the controls. This finding was emphasized by a complete disappearance of the sudanophobic zone (transition zone) which normally lies between the glomerulosa and fasciculata. This entire area and the adjacent fasciculata were filled with a large number of small, intensely staining lipid droplets. By the tenth day a decrease in the sudanophilia of the transition zone was observed and a normal sudanophobic zone was apparent by the sixteenth day (Fig. 1, *C*). The control and sham-operated animals presented a prominent sudanophobic zone throughout the entire experimental period. Since several investigators have described the lipid droplets of the zona fasciculata as fine when the gland is in a state of activity and much coarser during inactivity,[8, 9] these results seem to indicate an increased lipid activity in the fasciculata of the desalivated animals.

Birefringence.—No detectable differences in size and concentration of birefringent particles were noted in either the zona glomerulosa or zona reticularis of all three experimental groups. The zona fasciculata in the control and sham-operated animals had a predominance of coarse birefringent particles during the first 6 days, and about equal amounts of coarse and fine particles up to the thirty-second day (Figs. 2, *A* and 3, *A*). In contrast to either the control or sham-operated animals the fasciculata of the desalivated animals during the first six days had very fine birefringent particles (Figs. 2, *B* and 3, *B*). Thereafter they appeared similar to the controls. Since accumulating evidence suggests that coarse birefringence is indicative of inactivity or storage, and fine birefringence is indicative of activity or secretion,[7, 10, 11] these results seem to indicate that the fasciculata of desalivated animals is a very active zone during the 6 days following surgery.

Schiff Reaction.—The control and sham-operated animals had a strong Schiff reaction in the zona fasciculata and a considerably less intense reaction in both the glomerulosa and reticularis throughout the entire thirty-two days. The desalivated animals appeared to have an increased intensity of reaction in the zona fasciculata during the first six days, and thereafter were identical to

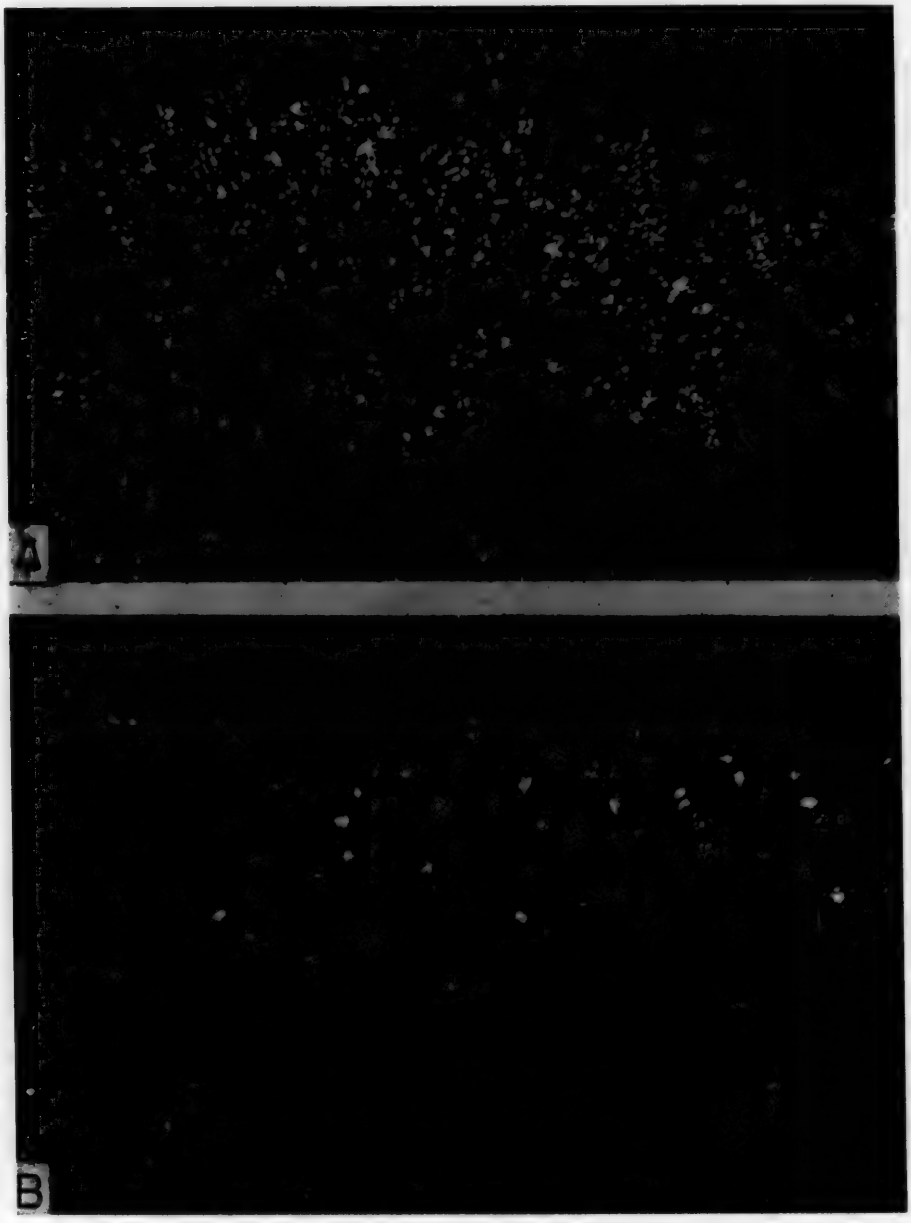

Fig. 2.—Adrenal cortices of rats illustrating the distribution and quantity of birefringent lipids. (Orig. Mag. ×100; reduced ⅙.) *A* is a control female at 48 hours on the experiment. In both control and sham-operated animals the birefringence is characteristically coarse. *B* is a desalivated female at the same period. Note fine birefringence in the fasciculata.

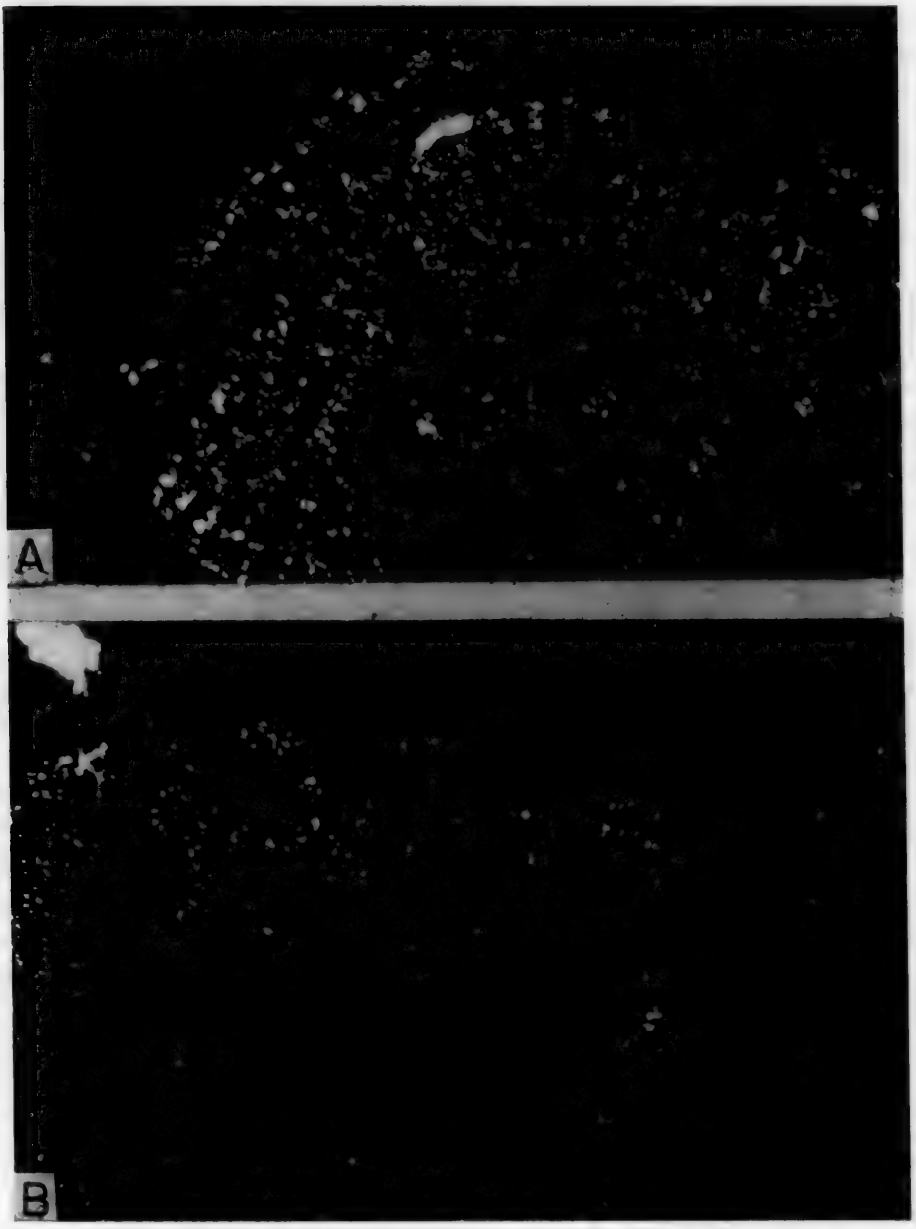

Fig. 3.—*A*, Birefringent properties of a control animal at the sixth experimental day. *B*, The persistence of fine birefringence in the desalivate rat's cortex. (Orig. Mag. ×100; reduced ⅙.)

the control animals. Since considerable question has been placed upon the specificity of this reaction,[12, 13] its interpretation is open to criticism. Nevertheless, the intensity of the plasmal reaction has been correlated to known functional states of the adrenal cortex[5, 7] and it is felt that the results described here indicate a more active fasciculata in the animals with their salivary glands removed.

Ascorbic Acid.—Both sexes of all three experimental groups showed the same distribution and concentration of ascorbic acid granules. Up to the sixth day these granules were present only in the glomerulosa and outer fasciculata, particularly the sinusoids of the latter. From the sixth to the twenty-fourth day the granules were evenly distributed throughout the fasciculata but still absent from the reticularis. By the thirty-second day a uniform distribution in all three cortical layers was noted.

Uterus and Ovaries.—The uteri of all three groups during the first 10 days were essentially hormonally unstimulated organs characterized by low columnar cells. The ovaries of these groups during this same period had follicles in all stages of development; however, none were mature and no corpora lutea were present. By the eighteenth day several corpora lutea were present in the ovaries of all three groups, although the desalivated animals characteristically had less than one-half the number of the control animals. The control uterus was definitely stimulated at this age showing a vascular and much thicker myometrium than it did 6 days previously, and a well-developed endometrium. The major difference between the control and desalivated uteri was in the degree of development of the whole organ. The desalivated animals had a much less well-developed endometrium and the whole organ was considerably smaller, so that control and desalivate uteri could readily be identified by gross inspection. These same differences between control and desalivated ovaries and uteri prevailed throughout the last 16 experimental days. The differences between control and desalivated ovaries and uteri were quantitative and not qualitative.

DISCUSSION

The data on birefringence, the Schiff reaction, and Sudan stains all indicate a more active fasciculata in the adrenal cortex of the desalivated animals during the first 6 days. Thereafter, no significant differences were evident. It seems from these data that the removal of the major salivary glands is accompanied by an increased adrenocortical activity particularly in the zona fasciculata. Upon a more critical examination of the cellular structure of this zone in the 3-day desalivates it was noted that a marked cellular hypertrophy was apparent. The cells were two to four times larger than those of the control and sham-operated animals and many appeared frothy and vacuolated. No increase in the number of cell mitoses was observed and there were no apparent evidences of hyperplasia. This same condition prevailed to the sixth day at which time the cortices of all three groups were essentially identical.

From the sixth to the thirty-second day no differences between any of the groups were observed. Baker[8] has reviewed the effects of ACTH on the adrenal cortex in hyperadrenocorticalism and observations made here of (1) cytochemical alterations, (2) cellular hypertrophy, and (3) no increase in mitotic activity in desalivated animals are all indicative of a moderately ACTH-stimulated gland.

SUMMARY AND CONCLUSIONS

The adrenal glands of desalivated, sham-operated, and control Sprague-Dawley strain rats were studied histochemically at various ages to determine their functional activity. The uteri and ovaries were examined histologically for any alterations which might accompany desalivation.

During the first 6 days after surgery, activity of the adrenal cortex of the desalivated animals increased. This was evidenced by the intensity of the Schiff reaction, and by conspicuously fine birefringence. Similarly, the Sudan stains indicated an increased lipoid activity during the same period in the desalivated animals, and the marked cellular hypertrophy of the cells of the fasiculata indicates an ACTH-stimulated gland. The control and sham-operated animals did not have any of these same alterations. From the tenth to the thirty-second day no differences were evident between any of the three experimental groups.

The uteri and ovaries of the desalivated animals revealed no pathologic alterations although the uterus of the desalivate was characteristically a considerably smaller and less well-developed organ than that of the control or sham-operated animals at the same age. From the sixteenth day to the termination of the experiment, the ovaries of the controls and sham-operated animals had two to three times as many corpora lutea as the desalivated animals.

The results of this study indicate that the removal of the major salivary glands is accompanied by an increased adrenocortical activity. Also, the development of the female reproductive system is retarded in desalivated animals.

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THE SEQUENCE OF CALCIFICATION OF THE MANDIBULAR MOLAR AND PREMOLAR TEETH

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INTRODUCTION

THE sequence of eruption of the permanent teeth has been the subject of considerable investigation both in man and in other primates. Though longitudinal data are available on but few individuals in any of the genera, the more usual sequences have been pieced together from cross-sectional studies, from skeletalized material, and from the investigation of fossilized remains.

For recent whites the eruption sequence given by Schultz,[1] namely, M_1 I_1 I_2 (P_1 C P_2) M_2, may be taken as representative. It matches the sequence of mean eruption times given by Fulton and Price,[2] and the means compiled by Hurme on 93,000 white children.[3, 4] Unaccountably, the data of Clements, Davies-Thomas, and Pickett on Birmingham (England) white children give the M_2 P_2 variant as the more likely for the mandibular teeth in both boys and girls.[5, 6] For races other than white the M_2 P_2 sequence has been reported,[7-9] and this latter sequence obtains in a number of fossil hominids,[10] as it does among the great apes in general.[1, 10, 11]

Little has been published on the sequence of calcification (the order of appearance of cusp or crown calcification) for these same teeth although Grewel[9] stated that the second molar begins to form in time between P_1 and P_2. Therefore, it is not possible to say whether the calcification sequence matches the eruption sequence either on an individual or on a group basis. Moreover, the extent of racial differences, prior to eruption, and the differences between living and fossil hominids in the sequence of tooth calcification have rarely been explored in any systematic way.

It is the purpose of this paper to provide new data on the sequence of calcification of the mandibular molar and premolar teeth in contemporary American white children. Further, it is our purpose to demonstrate the existence of several distinct calcification sequences and to provide evidence that these different sequences are familial in nature and very likely are genetically determined.

METHODS AND MATERIALS

The present study is based on serial oblique-jaw x-rays of 359 white boys and girls aged 1 month to 15 years. These subjects, native-born residents of

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southwestern Ohio, were all participants in the Fels Longitudinal Studies and had been examined at regular intervals since birth. A few children with marked growth disturbances have been excluded from the investigation. For obvious reasons children congenitally lacking P_2 were not included in the final tabulations on the sequence of calcification.

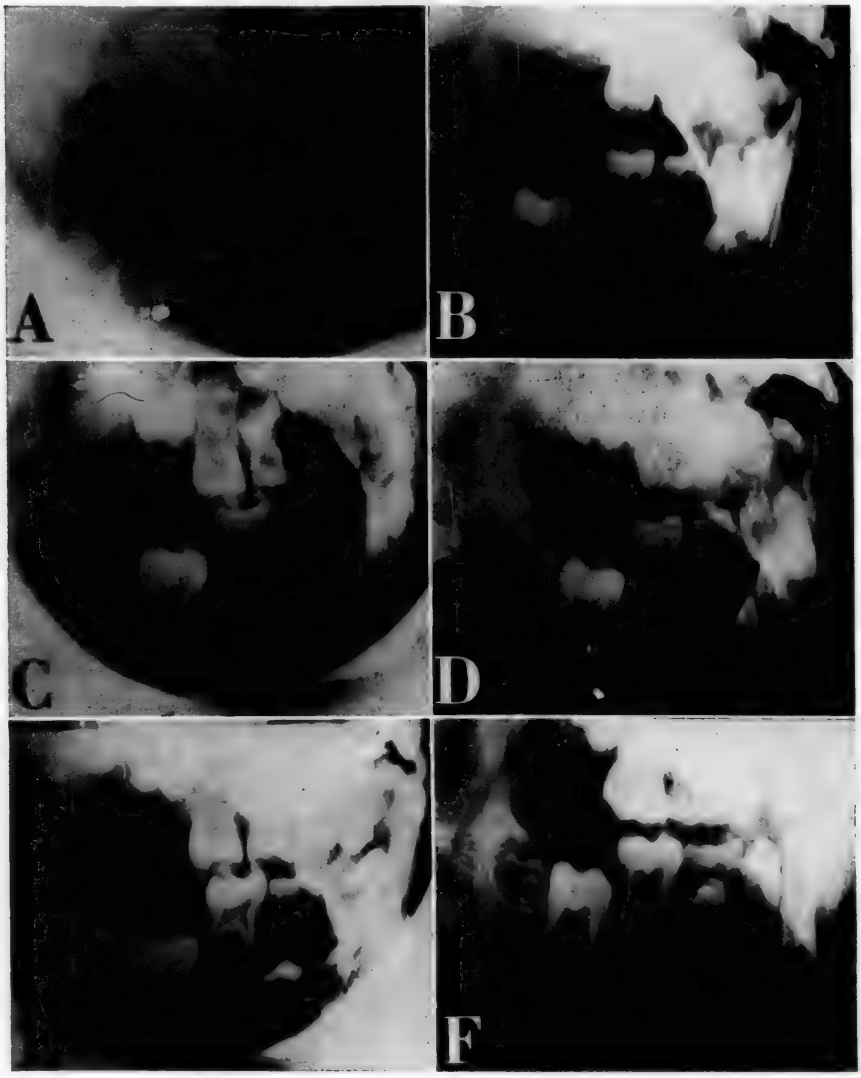

Fig. 1.—Reproduction of oblique-jaw x-rays showing calcification sequences described in the text. *A*, M_1. M_1 calcification evident, no other permanent teeth. *B*, M_1 P_1. M_1 calcification well advanced, early P_1 calcification. *C*, M_1 P_1 P_2 M_2. P_2 calcification extending to entire crown, M_2 cusp calcification only. *D*, M_1 P_1 (P_2 M_2). P_2 and M_2 equally advanced. *E*, M_1 P_1 M_2 P_2. M_2 calcification extending to entire crown, P_2 calcification at cusp coalescence stage. *F*, M_1 P_1 P_2 M_2 M_3.

The order of calcification of the mandibular molar and premolar teeth was determined for each child, primarily by reference to the age at which beginning (cusp) calcification was first noted for each tooth. Thus, if M_1 calcification was observed at 1 month by x-ray, P_1 calcification at 24 months, P_2 calcification at 30 months, and M_2 calcification at 36 months, the sequence was written as M_1 P_1 P_2 M_2.

Not infrequently, however, 2 teeth showing no sign of calcification on one regularly scheduled x-ray both evidenced calcification on the next regular film. In such cases it was often possible to assign priority by referring to the extent of calcification. Thus, if at 36 months M_2 was represented by seemingly complete crown calcification, and P_2 as only a slightly calcified cusp, the sequence M_2 P_2 could be assigned (Fig. 1). When 2 teeth appeared equally advanced in calcification, no attempt was made to assign priority. In such cases it could only be said that the order of calcification of a given second molar or second premolar tooth was either third or fourth, and in writing the sequence these "tied" teeth were bracketed, as is commonly done in recording the eruption sequence.

Although serial oblique-jaw x-rays were taken at 1, 3, 6, and 9 months following birth, and at birthdays and "half-birthdays" thereafter (within narrow tolerance limits), a complete series ranging from beginning calcification of M_1 to beginning calcification of M_3 was not available for all subjects. The older children in the Fels Studies were well along in childhood when the present jaw series was initiated, while the youngest children are still infants. This study therefore is semilongitudinal in nature and the number of cases used in each part of the analysis is given in the appropriate table.

It should be clearly understood that the present study refers to tooth calcification as seen in x-rays, and not to emergence or eruption. It is limited to those 5 teeth best visualized in oblique-jaw x-rays, which are superior to lateral-head x-rays for this purpose since there is no superimposition.

FINDINGS

As shown in Table I, 3 of the 5 mandibular molar and premolar teeth appear to hold unvarying positions in the calcification sequence. M_1 was first, in all of 293 cases observed, M_3 was invariably last in 140 cases, and among 202 cases P_1 was always second to calcify of these 5 mandibular teeth.

In contrast, P_2 and M_2 both exhibited considerable variability. In some children P_2 was third, and in others fourth. Conversely, M_2 calcification appeared third in some children, and fourth in others. In a large number of cases (145), P_2 and M_2 calcification was noted for the first time in the same x-ray, and without evidence as to which should be assigned priority (Table I).

There was a definite tendency for P_2 to appear third more often than fourth (69 vs. 43 cases) and for M_2 calcification to appear fourth more frequently than third (76 vs. 47 cases). Furthermore there was evidence that the order of calcification was influenced by sex. Thus the precedence of P_2

calcification over M_2 was more clearly defined in the girls than in the boys. "Bunching" was significantly more prevalent in the boys (44.9 per cent) as contrasted with girls (31.4 per cent).

TABLE I
ORDER OF CALCIFICATION OF MANDIBULAR MOLARS AND PREMOLARS

TOOTH AND SEX	FIRST NO.	FIRST PER CENT	SECOND NO.	SECOND PER CENT	THIRD NO.	THIRD PER CENT	THIRD-FOURTH NO.	THIRD-FOURTH PER CENT	FOURTH NO.	FOURTH PER CENT	FIFTH NO.	FIFTH PER CENT	TOTAL
P_1 Boys			106	100.0									106
P_1 Girls			96	100.0									96
P_1 Combined			202	100.0									202
P_2 Boys					28	30.1	43	46.2	22	23.7			93
P_2 Girls					41	45.1	29	31.8	21	23.1			91
P_2 Combined					69	37.5	72	39.1	43	23.4			184
M_1 Boys	153	100.0											153
M_1 Girls	140	100.0											140
M_1 Combined	293	100.0											293
M_2 Boys					25	25.3	43	43.4	31	31.3			99
M_2 Girls					22	22.7	30	30.9	45	46.4			97
M_2 Combined					47	24.0	73	37.2	76	38.8			196
M_3 Boys											67	100.0	67
M_3 Girls											73	100.0	73
M_3 Combined											140	100.0	140

Among the 191 boys and girls, for whom the entire sequence of calcification could be determined, the M_1 P_1 P_2 M_2 M_3 sequence, characterized by precedence of P_2 over M_2, was observed in 40.3 per cent. In 37.7 per cent the M_1 P_1 (P_2 M_2) M_3 sequence was assigned, since P_2 and M_2 calcification appeared simultaneously. In 21.9 per cent the M_1 P_1 M_2 P_2 M_3 sequence was manifest, with M_2 earlier to calcify than P_2. Thus less than half of the children followed the simple M_1 P_1 P_2 M_2 M_3 sequence that might have been expected on the basis of the eruption means. The extent of sex differences in the calcification sequence is shown in Table II.

TABLE II
FREQUENCY OF THE DIFFERENT CALCIFICATION SEQUENCES IN BOYS AND GIRLS

SEQUENCE AND CHARACTERISTIC	BOYS NO.	BOYS %	GIRLS NO.	GIRLS %	TOTAL NO.	TOTAL %
M_1 P_1 P_2 M_2 M_3 P_2 M_2	31	32.3	46	48.4	77	40.3
M_1 P_1 M_2 P_2 M_3 M_2 P_2	21	21.9	21	22.1	42	21.9
M_1 P_1 (P_2 M_2) M_3 (P_2 M_2)	44	45.8	28	29.5	72	37.7*

*Sex differences not significant at $p = .05$. The male and female distributions are significantly different, however, by the X^2 test.

TABLE III
FREQUENCY OF THE DIFFERENT CALCIFICATION SEQUENCES IN SIBLINGS OF CHILDREN EXHIBITING EACH OF THESE SEQUENCES

SEQUENCE IN INDEX CASES	SEQUENCE IN SIBLINGS: P_2 M_2 NO.	P_2 M_2 %	(P_2 M_2) NO.	(P_2 M_2) %	M_2 P_2 NO.	M_2 P_2 %	TOTAL NO.
P_2 M_2	26	66.6	10	25.6	3	7.7	39
(P_2 M_2)	18	41.9	13	30.2	12	27.9	43
M_2 P_2	6	27.3	6	27.3	10	45.5	22*

*All three distributions significantly different at $p = .05$ or better by the X^2 test.

While these different sequences P_2 M_2 (P_2 M_2) and M_2 P_2 might be attributed to chance, sibling parings suggested that other factors were operative (Table III). Siblings paired with children exhibiting the P_2 M_2 sequence followed this sequence more often than would be expected on the basis of the population figures (66.6 per cent as compared to 40.3 per cent). Among siblings of the (P_2 M_2) children, the P_2 M_2 sequence was less common and the M_2 P_2 sequence more common (Fig. 2). Siblings of M_2 P_2 children showed an increased proportion with the same calcification sequence (45.5 per cent as compared with a population percentage of 21.9 per cent).

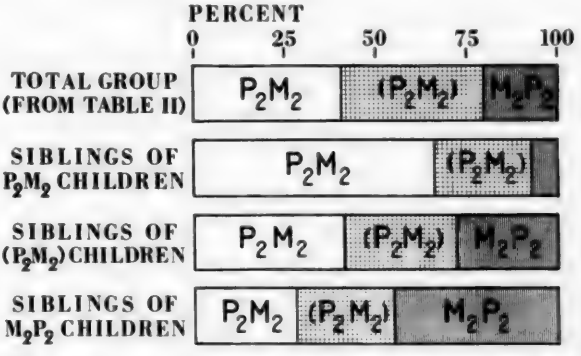

Fig. 2.—Bar graphs showing frequency of P_2 M_2 (P_2 M_2) and M_2 P_2 calcification sequences in siblings of children with the P_2 M_2 (P_2 M_2) and M_2 P_2 sequences, respectively. There is evidence for a tendency for the P_2 M_2 and M_2 P_2 sequences to "pile up" in particular family lines.

DISCUSSION

It is evident from the findings that there are 3 distinct sequences of calcification characteristic of the mandibular molar and premolar teeth, and that these sequences depend upon whether calcification of P_2 precedes, follows, or is simultaneous with beginning calcification of M_2.

Percentagewise, the M_2 P_2 sequence occurred least often, yet appeared in 22 per cent of both boys and girls. The P_2 M_2 and (P_2 M_2) sequences were almost equally common, and there was evidence that concurrent calcification of P_2 and M_2 tends to be a male characteristic. In beginning calcification, as in tooth emergence, "bunching" of these 2 teeth[2] appears to be a male trait. In some undetermined way, sex influences the timing of tooth calcification, both in the dentition as a whole, and differentially for different classes of teeth.

The evidence accumulated in this study does not support the idea that the different calcification sequences are merely chance combinations such as the combination of an early calcifying second molar and a late calcifying second premolar. Rather the P_2 M_2 and M_2 P_2 calcification sequence tends to pile up in family lines, suggestive at least of genetic control. It may be of some interest to note that children in the Fels series congenitally lacking P_2 tend to come from M_2 P_2 families, almost as though late P_2 calcification were

the partial expression of a trait characterized by total suppression when fully expressed. This presents a new alternative to the concurrently accepted idea that reduced tooth size represents the partial expression of a gene favoring complete suppression.[12]

Since the relationship between the sequence of calcification and the sequence of eruption has not been worked out, either on an individual or a group basis, it is obviously premature to link them. Needless to say, there is no certainty that a child who is M_2 P_2 in the calcification sequence might not appear (P_2 M_2) or even P_2 M_2 in tooth emergence. However, we should note that 80 per cent of the children in this study conform in their calcification sequence to the sequence of emergence considered "typical" for whites in general, and characteristic of Indians as well.[13]

The M_2 P_2 calcification sequence, however, is reminiscent of the emergence sequence typical of anthropoids, and reported for some fossil men, and for some nonwhite populations.[1, 10, 11] In contrast to what has been written, it suggests that there has been no "sharp break" between the anthropoids and fossil man on the one hand and recent man on the other, but rather a gradual evolutionary shift in the direction of precedence of P_2 over M_2, with differential rates of evolution in different hominid groups. The fact that some of the reports on tooth "emergence" in nonwhites are actually based on radiographs of skeletalized material makes a further discussion impractical at this time.

The value of the longitudinal method to dental research is demonstrated in this study. Quite obviously the results obtained would have been very difficult to achieve through the cross-sectional investigation of even a much larger sample of children. Furthermore, treatment of the data in the usual way, computing means and standard deviations, and stressing teeth rather than children, would have lost the (P_2 M_2) and M_2 P_2 sequences entirely. Problems involving sequence and patterning during growth and development are best approached longitudinally and with the inclusion of siblings in the sample under consideration.

SUMMARY

1. The sequence of calcification of the mandibular molar and premolar teeth was investigated in serial oblique-jaw x-rays of 359 native-born white children from southwestern Ohio.

2. M_1 P_1 and M_3 invariably showed initial calcification first, second, and last, respectively. P_2 and M_2 calcification varied in the order of appearance, and in many cases these teeth began to calcify at the same time.

3. In the girls the tendency for P_2 to precede M_2 was more marked. The boys, however, tended to the (P_2 M_2) sequence, thus exhibiting "bunching" in tooth calcification as they are known to do in tooth emergence.

4. Among 191 boys and girls, the M_1 P_1 P_2 M_2 M_3 sequence was observed in 40.3 per cent, the M_1 P_1 (P_2 M_2) M_3 sequence in 37.7 per cent, and the M_1 P_1 M_2 P_2 M_3 sequence in 21.9 per cent.

5. Siblings of P_2 M_2 children tended to the P_2 M_2 sequence and siblings of M_2 P_2 children to the M_2 P_2 sequence more often than would be expected on a chance basis.

6. It was suggested that there has been a gradual evolutionary change from the M_2 P_2 sequence in the direction of the P_2 M_2 sequence, with a different rate of evolution in different hominid groups.

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FURTHER STUDIES ON THE EFFECT OF PROLONGED SLEEP ON THE ERUPTION OF THE RAT INCISOR

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THE effect of prolonged sleep on the rate of eruption of the incisor of the albino rat held on suboptimal nutritional regimen has been described.[1] It has been shown that there are several methods capable of inducing accelerated eruption of the rat incisor, i.e., dissection of the inferior alveolar nerve,[2-4] repeated cutting of the incisal edge,[5] dissection of the cervical sympathetic nerve,[6] changes in the consistency of food,[7, 8] use of protracting and retracting rubber bands. The most marked increase has been attained by repeated cutting of the incisal edge. It appeared worth while to ascertain what happens under similar experimental conditions to the incisor of rats in whom prolonged sleep is induced by administration of drugs. In our first series, animals were not optimally nourished and were deficient in weight in comparison to rats fed optimally. Possibly, for this reason, the eruption rate of our controls was less than data in the literature indicated as being normal. Therefore it was necessary to control our previous findings in rats fed optimal diets.

MATERIAL AND METHODS

Mature albino rats of both sexes, equal age, and held since birth under identical conditions, were used. The diet, a formula* of the Hungarian State Institute of Nutrition, was given by placing a quantity into the cages exceeding the daily consumption. The food was taken ad libitum. Fresh food was placed into the cages daily.

Prolonged sleep was induced by subcutaneous injections of a mixture of 8 per cent paraldehyde and 8 per cent ethyl urethane dissolved in distilled water. One injection was given daily. Forty milligrams of each of the drugs was given for 100 Gm. of body weight. This dosage is well tolerated.[1]

The eruption rate of the incisor was ascertained by marking, at weekly intervals, all 4 incisors in all rats with a sharp cutting dental disc at the level of the gingival margin and by measuring the distance between 2 subsequent markings with precision calipers permitting an accuracy of 0.01 mm. In all animals the opposing upper and lower incisor of one side was ground as much as possible without injuring the pulp, at intervals of 1 week. In one-half of the animals the left and in the other half the right incisors were cut in this manner. Measurements were carried out in 30 animals through an

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*The finely ground mixture of 20 per cent wheat grains, 20 per cent rye gains, 20 per cent barley grains, 15 per cent corn grits, 10 per cent oat grains, 8 per cent oily seeds, 8 per cent casein, 1 per cent calcium lactate, 0.5 per cent NaCl, and 0.5 per cent dried alfalfa mixed with water to form a pap (Tarján, Sásdi, and Dénes[9]).

experimental period of 4 weeks. The first marks were made in the teeth of the experimental animals 4 days after having started with the administration of the narcotic drugs. Results of measurements were subjected to elementary statistical analysis.

FINDINGS

Results of measurements are summarized in Table I. In comparing the weekly growth rates, as well as the combined results of the 4-week experiment, a marked difference between experimental animals and controls is obvious. This difference is found in both the maxillary and mandibular incisors. The difference was statistically significant (Table II). The eruption rate of the intact (noncut) incisors was significantly greater in the experimental group compared with the controls. The results of our first report[1] thus were verified in optimally fed animals.

TABLE I
THE WEEKLY ERUPTION RATES OF UPPER AND LOWER, INTACT, AND CUT INCISORS IN EXPERIMENTAL AND CONTROL ANIMALS

			UPPER INCISORS		LOWER INCISORS	
			INTACT	CUT	INTACT	CUT
GROUP	WEEK	N	M ± e	M ± e	M ± e	M ± e
Exp.	I	24	2.51 ± 0.03	3.40 ± 0.03	4.04 ± 0.04	5.75 ± 0.03
Exp.	II	23	2.63 ± 0.03	3.67 ± 0.03	4.13 ± 0.03	6.31 ± 0.03
Exp.	III	22	2.40 ± 0.03	3.60 ± 0.04	3.88 ± 0.04	6.13 ± 0.03
Exp.	IV	22	2.78 ± 0.02	3.58 ± 0.02	3.68 ± 0.03	5.78 ± 0.03
Exp.	I-IV	91	2.59 ± 0.05	3.56 ± 0.04	3.98 ± 0.08	5.99 ± 0.07
Control	I-IV	16	2.33 ± 0.03	3.24 ± 0.06	3.13 ± 0.07	5.84 ± 0.04

N = number of measurements (animals).
M = mean of the weekly eruption rate in millimeters.
e = standard error of the mean.
Exp. = animals receiving daily injections of the paraldehyde–ethyl urethane mixture, 40 mg. of each drug for 100 Gm. weight.

TABLE II
STATISTICAL SIGNIFICANCE OF DIFFERENCES

ERUPTION RATES COMPARED			DIFFERENCE OF MEANS	STANDARD ERROR OF DIFFERENCE
Exp. intact upper inc.	vs.	Contr. intact upper inc.	0.26	± 0.06
Exp. cut upper inc.	vs.	Contr. cut upper inc.	0.32	± 0.07
Exp. intact lower inc.	vs.	Contr. intact lower inc.	0.85	± 0.10
Exp. cut lower inc.	vs.	Contr. cut lower inc.	0.61	± 0.08
Exp. cut upper inc.	vs.	Exp. intact upper inc.	0.97	± 0.06
Exp. cut lower inc.	vs.	Exp. intact lower inc.	2.01	± 0.10
Contr. cut upper inc.	vs.	Contr. intact upper inc.	0.91	± 0.06
Contr. cut lower inc.	vs.	Contr. intact lower inc.	2.71	± 0.08

The marked acceleration of the eruption rate resulting from cutting the edge of the incisor is explained adequately by loss of contact with antagonists during function, lessening the intruding forces that act upon the erupting tooth. According to Taylor and Butcher,[5] a 100 per cent increase in the eruption rate is observed if the incisor is cut at the edge at 2 to 4 days' intervals. In our experiments incisors were cut once weekly only. Thus, the incisors were not freed from contact with functioning antagonists through

the total experimental period in contrast to the experiments of Taylor and Butcher. For this reason a direct comparison of their increase and ours is not justified. According to our observations, there is no contact between the shortened incisors at the end of the third day after having been cut but after 4 days a definite contact could be seen. Therefore, it is assumed that the difference observed in the eruption rate of the intact and cut incisor of the same animals is due to differences in eruption during the first half of each week. In the second halves of all 4 weeks, opposing incisors are in contact in spite of having been cut. The eruption rate, during the second half of each week, presumably, is equal to that of incisors that were in contact with their antagonists during the whole experimental period. On the basis of these considerations, the weekly eruption rates were divided into 2 phases, one for the first and one for the second half of every week. The eruption rate should be equal in both phases for the intact (noncut) incisors and in the second phase for the cut incisor. The total difference observed is then transposed to the first halves. Results of this computation are summarized in Table III. In comparing the eruption rates in the first phases between intact and cut incisors, differences of equal range are obvious as in the experiments of Taylor and Butcher.[5]

TABLE III
HALF-WEEKLY ERUPTION RATES OF INTACT AND CUT INCISORS

HALVES OF THE WEEKS	TOOTH	GROWTH IN MM. OF INCISORS		GROWTH OF CUT INCISOR IN PER CENTS OF THE INTACT ONE	PERCENTAGE INCREASE OF ERUPTION RATE
		CUT	INTACT		
1. Control animals					
first	upper	2.075	1.165	178	78
second	upper	1.165	1.165	---	--
first	lower	4.275	1.565	273	173
second	lower	1.565	1.565	---	---
2. Experimental animals (prolonged sleep)					
first	upper	2.265	1.295	175	75
second	upper	1.295	1.295	---	--
first	lower	4.000	1.990	201	101
second	lower	1.990	1.990	---	---

Since eruption rates in second halves of every week are supposedly equal, the last two data in the horizontal rows are left empty as regards this period.

The increase of the eruption rate of maxillary incisors is approximately equal in experimental animals (prolongedly sleeping) and controls, whereas in the mandibular incisors a difference appears to exist. Further study is required to determine whether or not this is due to chance alone or to any particular heretofore unidentified factor.

By regarding the eruption rates as divided into 2 phases it seems to be apparent that the eruption rate is accelerated, both in the intact and in the cut incisor, by drug-induced prolonged sleep. The reason for the increased eruption rate in the cut incisor is a reduction of retrusive forces acting upon the tooth. Since the increase in the eruption rate which was observed for the cut incisors of experimental (prolongedly sleeping) and control animals was approximately equal, we are justified in assuming that the eruption-promoting

effect of prolonged sleep is not due to lessening of intrusive forces acting upon the incisors. This observation supports the conclusion derived from our first report,[1] i.e., that prolonged sleep acts as an accelerator of the eruption of the rat incisor per se, the action probably being due to the transitory partial paralysis of the brain.

SUMMARY

By prolonged sleep, induced by administration of a mixture of paraldehyde and ethyl urethane, the eruption rate of the incisor is significantly increased in rats fed optimally. The eruption rate of cut incisors (those free of intrusive forces) is increased to the same degree over that of the intact incisors in prolongedly sleeping animals and controls. This observation supports the view that increased eruption rate of rat incisors during prolonged sleep is not due to reduced intrusive forces acting upon the tooth.

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ELECTROMETRIC STUDIES OF HUMAN GINGIVA IN PREGNANCY

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CONNECTIVE tissues are labile in response to the influence of hormones and changes in the internal environment. This behavior is determined by changes in the state of aggregation of the negatively charged colloids, including the mucoproteins of the ground substance.[1-3] The oral tissues, especially the gingivae, participate in these responses of connective tissue.[3-5] The changes in the gingivae of women during pregnancy illustrate this phenomenon. There may be considerable swelling with the development of "pregnancy tumors," or less distinctive alterations such as redness, edema, and bleeding.[5-8] Since these signs do not appear in all pregnant women, their direct relation to the pregnant state has been questioned.[7] An objective study of this condition is facilitated by an electrometric method[2, 9] which determines the change in state of the connective tissue.

In a group of pregnant women examined in this way almost all showed evidence of disaggregation of the gingival connective tissues, though often no clinical changes could be observed. Return to the normal state occurred in the postpartum period. It is clear, then, that the gingival tissues participate in the generalized somatic reaction during pregnancy.

MATERIALS AND METHODS

A group of 41 women, Negroes and Caucasians, whose ages ranged from 13 to 35 years, was studied. The subjects were first tested during pregnancy and again at 4 to 8 months post partum. Only 21 of the 41 were available for re-examination after parturition. A control group of 14 nonpregnant women, whose gingivae appeared normal and showed no inflammatory changes, was also examined. The gingival area selected for testing was at the base of the interdental papilla between the mandibular left canine and first premolar teeth.

The experimental procedure follows that of Steck, Montgomery, Reed, and Joseph,[10] who measured intra-articular potentials in human knee joints. The apparatus consisted of 2 saturated KCl calomel half-cells, with NaCl leads extending from each cell to the experimental subject (Fig. 1). At the junction of the NaCl and KCl solutions, saturated KCl agar bridges were placed.

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One lead, closed with a .15 M NaCl agar bridge, was taped to the wrist and the contact moistened with a gauze sponge saturated with .15 M NaCl. This served as the reference junction. The other lead was adapted to fit the hub of a needle which was to be inserted into the experimental site. The schematic representation is given by: $Hg/Hg_2Cl_2/KCl/.15$ M NaCl/Skin/Gingiva/.15 M or .015 M NaCl/$KCl/Hg_2Cl_2/Hg$. The experimental site in each patient was anesthetized by means of an inferior alveolar block with a 2 per cent procaine solution containing 1:50,000 Adrenalin. A 20 gauge hypodermic needle, cut to approximately ½ inch length, was inserted into the basal portion of the papilla and maintained manually throughout the experiment. Initially, .15 M NaCl was instilled into the needle with a syringe using a 27 gauge needle. The junction with .15 M NaCl was formed, and the potential in millivolts was determined. This was followed by 2 irrigations with the same solution. When

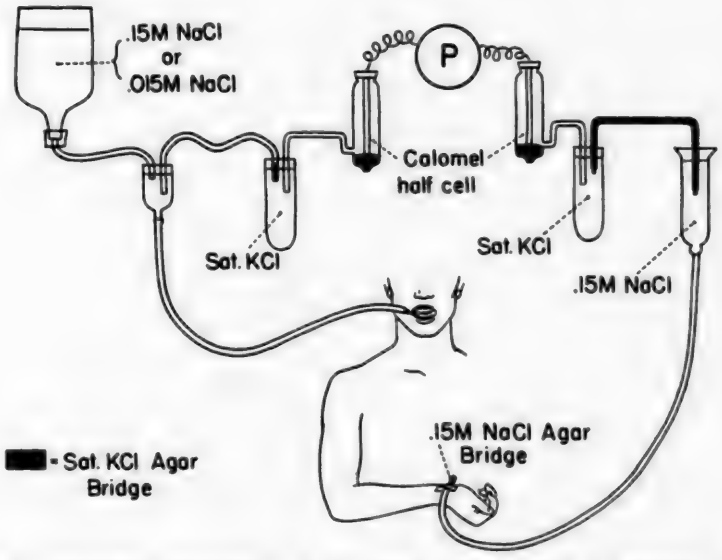

Fig. 1.—Schematic representation of the circuit used to obtain the dilution potentials. The base-line potential E is determined with .15 M NaCl. A displaced potential is determined with .015 M NaCl. The difference between the two is termed the dilution potential E_d.

3 readings agreed within 1 mv., the average was taken as the base-line potential, E. The .15 M saline was then withdrawn from the needle and replaced with .015 M NaCl, a 1/10 dilution of the isotonic saline. A junction with .015 M NaCl was formed in the gingival connective tissue and the displaced potential E′ was determined. The difference between E′ and E is termed the "dilution potential" E_d. The base-line readings were restored by replacing the dilute salt solution with the normal saline. Two or three flushings sufficed to obtain reproducible values. Upon return to the base-line potential, the experiment was completed.

The relation between E_d and the colloidal charge density x has been expressed by the approximation formula

$$E_d = -12.3 + 215x$$

Where E_d is expressed in millivolts and x in equivalents of immobile negative colloidal charge per kilogram of tissue water. The constant, −12.3 mv., represents the value of the junction potential between 0.15 M and 0.015 M NaCl solutions. The state of aggregation of the colloids of connective tissues has been related to the value of x.[2, 3, 9] Loose hydrated tissues, where colloidal density is low, give negative values for E_d, approaching −12.3 mv. as a limit. Dense tissues such as cartilage give positive values for E_d which may be as high as 25-30 mv., and values of x of .17-.20 equivalents per kilogram of water.

RESULTS

A summary of the results for the control and experimental groups appears in Table I and the distribution of dilution potential values is illustrated in the histograms of Fig. 2. The control group showed the highest and most uniform values. Gingival dilution potentials of this group ranged from +6.9 to +11.9 with a mean of +8.7 mv. This corresponds to a density of negatively charged colloid of 0.098 equivalent per kilogram of water. Tests of pregnant women yielded the lowest values, ranging from −2.8 to +5.4 with a mean of +1.6 mv., corresponding to a value of 0.065 equivalent for x. The postpartum potentials, with one exception, were all higher than the prepartum ones, and approached the control levels. The mean value was +5.4 with a range of −1.7 to +8.4 millivolts, the value of x being 0.082 equivalent. A comparison of the values obtained for the prepartum, postpartum, and control groups shows a highly significant difference ($P < .001$). Of the 41 pregnant women tested, 28 had clinically healthy gingivae, the tissues appearing firm and pink with

TABLE I
STATES OF THE GINGIVA IN NORMAL AND PREGNANT WOMEN

GROUP	NO. OF EXP.	MEAN DIL. POTEN. (± S.E.)		IMMOBILE CHARGE DENSITY* (MEQ./KG. H_2O)
Control	14	+ 8.7 ± .41	P <.001	97.7
Prepartum	41	+ 1.6 ± .24		64.6
Postpartum	21	+ 5.4 ± .43		82.3

*Calculated from $E_d = -12.3 + 215x$.

no bleeding or edema. Seven had mildly inflamed gingivae, while 6 patients showed severe involvement with redness, swelling, and great friability. In the presence of severe gingival involvement the potential values were at or below zero. In the postpartum period the greatest rise in the dilution potentials was observed in those subjects who showed no clinically visible involvement of the gingiva during pregnancy. The smallest change in the potential values was observed in those subjects who showed marked clinical involvement of the gingiva throughout the duration of the experiment. The distribution curve for the results in pregnancy and in normal women is of the normal

type, while that for the prepartum group appears to be bimodal. The two peaks are perhaps characteristic of a general population where one would find 2 groups, one having inflamed gingiva, due largely to local factors, and the other showing healthy tissues.

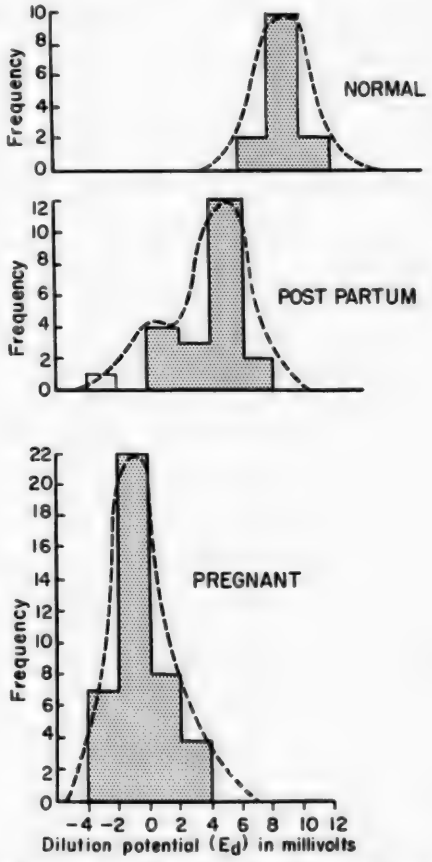

Fig. 2.—Histograms representing the distribution of the groups studied. The highest dilution potentials were obtained for the control group, while the lowest were those of the pregnant group. Values for the postpartum group approached those of the normal. The bimodal distribution of the postpartum series was interpreted to be representative of the general population where one group may have inflamed gingiva due largely to local factors and the other showing healthy tissues.

DISCUSSION

Connective tissue consists of cells and an extracellular colloidal matrix, formed of fibers and ground substance. Carbohydrate-protein complexes form important constituents of the ground substance, and can be identified in tissue sections by histochemical and chemical means.[1, 3, 11, 12] Such studies suggested that the carbohydrate-protein complexes exist as polymers, the physicochemical properties of the tissue depending on their state of aggregation.[1-3]

In loose tissue the ground substance is considered to be in a low state of aggregation, while in dense tissues such as cartilage and bone it is believed to be highly polymerized. The labile, reversible state of connective tissues depends on the state of aggregation of the ground substance which is mediated by cellular metabolism.

Then connective tissues are considered to behave as heterogeneous physicochemical systems which contain varying density of immobile negatively charged aggregates.[2, 3] The glycoproteins of the ground substance evidently form a substantial fraction of the charged colloid, which functions as a cation exchange resin.

It has been shown in experimental studies that hormones can produce changes in the colloidal properties of a variety of connective tissues, including the gingiva.[1-3, 5, 9, 12] By histochemical means, these were identified with alterations of the glycoprotein ground substance. An increase in the water-soluble fraction of the colloid leads to uptake of water and to the apparent binding of intravitally injected Evans blue.[1, 11] These observations are consonant with the idea that some hormones effect a depolymerization of the mucoproteins of the ground substance or stimulate the connective tissue cells to produce water-soluble components, leading to a redistribution of tissue water and electrolytes.

During pregnancy, endocrine activity may lead to swelling and edema of the gingiva, features of pregnancy gingivitis. Often the response may be more subtle, remaining occult and subclinical, and requiring sensitive electrochemical or histochemical methods in order to be demonstrated. Similar responses might be expected during the puberal period and during certain stages of the menstrual cycle. In any of these states a superimposed stress, such as poor hygiene, faulty occlusion, or general illness may accentuate the condition to the point of clinical manifestation.

The change in the density of colloidal charge in gingival connective tissue resembles the process observed in the pubic symphysis of the guinea pig also during pregnancy.[2, 12] The changes in tooth mobility during pregnancy observed by Mühlemann seem also to have a similar basis.[13] The tissue response in pregnancy can be regarded as a specific instance of an adaptation of the gingiva induced by hormones.

SUMMARY

Changes in the gingival connective tissues during pregnancy have been demonstrated using an electrometric method. The density of negatively charged colloid, including the glycoproteins of the ground substance, was decreased in the early months of pregnancy. The tissues were restored to the normal state several months after parturition. The connective tissue alterations, present in almost all patients studied, were often unaccompanied by any clinical signs. This response of the gingiva is an example of the sensitivity of oral tissues to hormones or other somatic influences.

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A STUDY OF THE BACTERIAL FLORA OF CARIES-SUSCEPTIBLE AND CARIES-IMMUNE SALIVA

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INTRODUCTION

SEVERAL types of bacteria have been associated with the etiology of caries, most commonly streptococci and lactobacilli, which are both acidogenic and relatively aciduric. Streptococci are more numerous in saliva and plaques[1, 2] but lactobacilli are capable of producing a lower pH in media. Furthermore, the relationship between high numbers of salivary lactobacilli and low pH minima in plaques,[3] and the correlation between the continued presence of relatively high numbers of salivary lactobacilli and caries activity associate these organisms with the decay process.[4] Considerable work has been done to determine whether streptococci or lactobacilli are more intimately associated with caries, and the work has been recently reviewed by Rosebury[5] and Harrison.[6]

A number of adults apparently completely resistant to caries, have been termed "immune" since they have no history of this disease. This immunity has not been associated with age, sex, race, or dietary habits, but there is some evidence suggesting a genetic factor in caries resistance.[7] In terms of the acidogenic theory of caries, it must be assumed that the organisms responsible for caries in susceptible persons are absent from immune mouths, or that the acidogenic activities of such organisms are either modified or neutralized in immune mouths. This last would imply the operation of some mechanism in the immune mouth which inhibits or modifies the activities of potentially cariogenic organisms. It has been previously shown by several investigators that salivary lactobacillus counts in immunes are very low and may be nonexistent in many subjects,[4, 8] while counts of streptococci are nearly the same in both immune and caries-susceptible salivas.

The purpose of the investigations presented here is to compare the salivary microflora of caries immunes and susceptibles and to study the effect of saliva upon certain oral organisms in vitro, for indications of possible mechanisms of immunity to dental caries.

MATERIALS AND METHODS

The adults whose salivas were studied were divided into 2 groups: caries immunes and caries susceptibles. The caries immunes had no evidence of

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caries history on the basis of clinical and roentgenologic examinations. Clinical and radiographic examinations were made of this group at intervals to check on the maintenance of the immune state. The caries-susceptible group was selected only on the evidence of past caries activity disclosed by clinical examination.

Saliva for bacteriologic examination was collected in sterile 10 ml. screw-cap bottles containing two glass beads. Paraffin was used for rapid production of saliva. All samples were collected no less than 1 hour after a meal, and the subjects were requested to refrain from smoking and chewing gum for 10 minutes prior to collection.

A number of media and supplements were employed for cultivation of salivary microorganisms. Tomato juice agar was prepared according to the method of Hadley,[4] containing 0.0004 per cent bromeresol green and 0.0001 per cent sodium azide.

Saliva which was added to media was pooled saliva obtained by continuous collection from 6 to 12 subjects. The pooled sample was held at 4° C. overnight, then centrifuged for 10 minutes at 2,000 r. p. m. for gross separation of the heavy mucinous precipitate. The supernatant was filtered through Whatman No. 1 paper in a Buchner funnel, using suction. The resulting filtrate was passed through a Seitz bacteriologic filter and stored in rubber-stoppered vaccine bottles at –10° C. These operations were performed either in the cold or with chilled equipment. Filtered saliva was tested for sterility by inoculating 1 ml. into thioglycollate broth and incubating at 37° C. for 7 days.

When a sample of fresh saliva was to be examined bacteriologically, the bottle was shaken in a Kahn shaking machine for 5 minutes. Dilutions of the saliva were made in 0.9 per cent saline and mixed by pipette; then 0.1 ml. of the proper dilution was placed on the surface of an agar medium and distributed over the surface with a bent glass rod.

All cultures were incubated at 37° C. After a suitable period, the colonies on the plates were examined with the aid of a wide-field microscope binocular at 7 and 10 magnifications. Turbidimetric measurements were made with an Evelyn colorimeter, using a 620 mμ filter with colorless solutions and a 540 mμ filter with light brown solutions. The substrates used in turbidimetric studies were made as follows: (1) filtered saliva diluted 1:1 with saline, (2) filtered saliva diluted 1:1 with saline containing 1 per cent glucose, and (3) filtered saliva diluted 1:1 with tomato juice broth pH 7.0. Anaerobiosis was obtained with Brewer electric anaerobic jars. Estimation of acid production was visual, except where otherwise noted.

RESULTS

The first part of this investigation was a comparative study of the salivary microflora of caries-immune and caries-susceptible individuals. Saliva samples were obtained from 25 subjects in each group, and examined by plating on several media. In selecting types of organisms to be enumerated, emphasis was placed upon types whose physiology would suggest them for

a place in the caries process. Otherwise, no attempt was made to differentiate genera or species, and comparison was made on the basis of the groups listed in Table I. Colony counts of acidogenic streptococci and micrococci on tryptone glucose extract agar were much lower than the counts of these obtained on blood agar, and the latter were accepted as the total counts. Counts of

TABLE I
MEDIAN COUNTS OF SALIVARY MICROORGANISMS FROM 25 CARIES-FREE AND 25 CARIES-SUSCEPTIBLE HUMAN BEINGS

ORGANISMS	IMMUNE	SUSCEPTIBLE
Gm. neg. diplococci	660,000	400,000
Gm. neg. rods	100	100
Caseolytics	100	100
Yeasts	100	100
Streptococci	30,000,000	50,000,000
Micrococci	900,000	1,000,000
Lactobacilli		
Aerobic	60	8,000
Anaerobic	400	18,000

	MEANS AND STANDARD ERRORS			
	IMMUNE	SUSCEPTIBLE	"t"	PROBABILITY
Gm. neg. diplococci	5,160,000 ± 4,048,000	1,278,333 ± 450,000		
Gm. neg. rods	336 ± 200	1,672 ± 1,200		
Caseolytics	68 ± 8	60 ± 6		
Yeasts	25 ± 17	258 ± 128	1.806	0.08
Streptococci	27,940,000 ± 3,560,000	38,584,000 ± 3,410,000		
Micrococci	3,528,000 ± 1,580,000	5,448,000 ± 2,700,000		
Lactobacilli				
Aerobic	858 ± 290	46,960 ± 16,600	2.77	0.009
Anaerobic	1,081 ± 314	399,960 ± 200,000	1.99	0.05

acidogenic organisms, though not presented, were not statistically different in immune and susceptible saliva. Counts of lactobacilli, streptococci, micrococci, gram-negative diplococci, and acidogens were made both aerobically and anaerobically. In order to avoid any possible inhibitory property of immune saliva, counts were made with diluted and whole saliva. No significant difference was found in the totals.

In Table I the most striking difference lies in the numbers of lactobacilli. Some of the data were analyzed by the "t" test, and the difference was shown to be significant. The counts of yeast are not significantly different, despite the tenfold difference in the means. When counts were made from saliva – brain heart infusion broth dilutions (1-10), after 5 hours incubation, the saliva dilutions showed no difference between immune and susceptible flora with reference to the original counts.

Organisms other than lactobacilli were at times observed growing on tomato juice agar (pH 5). These were identified as aciduric streptococci which grew from nearly all the immune salivas, and from fewer of the susceptible samples. This confirms the work of Snyder,[8] who also found higher numbers of aciduric streptococci in caries-free mouths. The significance of this finding is not apparent. Aciduric micrococci were observed infrequently from both groups.

Hadley, Bunting, and Delves[9] described several morphologic types of oral lactobacilli, and varieties of the types they considered most common. Barber and Frazier,[10] studying lactobacilli of nonoral origin, distinguished 5 colonial types of morphology, ranging from smooth to rough. In this work, colony morphology has been differentiated into 3 general types as recognized on tomato juice agar at pH 5, which has been used as the reference medium for colonial morphology of lactobacilli throughout this study. These are (1) smooth S, in which the colony is round, translucent to opaque, convex, and glistening; (2) rough R, which is flat, with serrate margin, and a rough and dull appearance. Some rough colonies have a smooth convex center, the whole resembling a fried egg. (3) Intermediate I, which is nearly a composite of smooth and rough characteristics. This colony is convex and has a serrated margin and a dull to glossy surface, while the texture is nonhomogeneous, presenting a curdled appearance. On the reference medium these 3 types of colonies may possess a green to greenish-white color, due to the presence of bromcresol green in the medium.

TABLE II
PERCENTAGE OF ROUGH COLONIES IN THE TOTAL LACTOBACILLUS COUNTS OF SALIVAS

SUBJECT NUMBER	IMMUNES		SUBJECT NUMBER	SUSCEPTIBLES	
	AEROBIC	ANAEROBIC		AEROBIC	ANAEROBIC
1	—	—	1	51.2	29.4
2	0	0	2	62.5	100
3	0	0	3	—	100
4	0	—	4	92.5	99.9
5	—	—	5	88.0	100
6	0	0	6	—	—
7	0	0	7	—	—
8	0	0	8	0	100
9	0	0	9	0	100
10	0	0	10	65.0	36.2
11	—	—	11	100	100
12	—	—	12	—	93.5
13	—	—	13	77.0	38.0
14	—	—	14	100	45.0
15	0	0	15	0	0
16	0	0	16	0	0
17	0	0	17	100	100
18	47.4	0	18	—	—
19	0	0	19	—	—
20	0	0	20	—	100
21	—	—	21	—	45.9
22	0	100	22	0	—
23	—	—	23	94.6	—
24	—	—	24	100	85.7
25	—	—	25	—	100

— = No colonies appeared.

The data in Table II show that the rough type of lactobacillus was seen almost never on plates from immune salivas, while 55 per cent of the colonies developing from susceptible salivas were of the rough type.

In exception to the almost exclusive association of smooth lactobacilli with immune individuals, 2 subjects had been found with no clinical evidence

of caries activity from whose saliva rough and intermediate types could occasionally be isolated. These organisms, though not common to the immune group, have been designated immune R and I types.

Various cultures of lactobacilli isolated from immune and susceptible saliva were maintained on tomato juice agar at pH 5 with frequent transfers for some time without change in colonial morphology. The capacity of these organisms to grow in a number of laboratory media was established (Table III). The susceptible R types were more exacting than the other types, while I types fall between the S and R types in relative fastidiousness. The appearance of growth of these cultures in liquid media exhibited another difference, the S types and immune R types were sedimented, while the I types and susceptible R types were diffused through the liquid.

TABLE III
GROWTH OF COLONY TYPES OF LACTOBACILLI IN VARIOUS LABORATORY MEDIA

	SOURCE AND TYPE OF ORGANISM																			
	IMMUNE							SUSCEPTIBLE												
MEDIUM	S	S	S	I	R	R	R	S	S	S	I	I	R	R	R	II	II	R	R	R
Tomato juice agar	4	4	4	4	4	4	3	4	4	4	4	4	4	3	4	4	3	4	4	4
TGE agar	4	4	4	3	3	3	2	4	3	4	2	2	–	–	–	–	–	–	–	–
Heart infusion broth:																				
Without additives	3	3	3	+	+	–	–	3	2	3	+	–	–	–	–	–	–	–	–	–
+ glucose	3	3	3	+	2	–	+	3	2	3	+	–	–	–	–	–	–	–	–	–
+ glucose + tryptone	4	3	4	2	3	3	2	4	3	4	3	–	–	–	–	–	–	–	–	–
Tomato juice broth:																				
Without additives	4	4	4	4	4	3	3	4	4	4	4	2	+	+	4	2	2	2	2	3
+ tryptone				4			3		4			4			4	4	4	4	4	4
+ egg albumin												2			4	2	2	2	2	3
+ Tween 80												4			4	4	4	4	4	4
+ trypticase + yeast extract + 2% glucose	4	4	4	4				4	4	4			2	2	3	3	4	2	2	

– = No growth.
+, 2, 3, 4 = Degree of growth.

The acidogenic capacity of the cultures was determined in several media. The data in Table IV show that the susceptible R organisms were less acidogenic than the other types. In tomato juice broth, immune and susceptible R strains were about equally acidogenic, but on tomato juice agar the difference is marked.

The aciduric properties of a number of lactobacillus cultures were investigated by 2 methods. In one experiment, the organisms were inoculated into tomato juice agar plates at varying pH, and the results showed that all the cultures tested grew at pH 5.0, 7.0, and 8.0 without any change in colony morphology, while only the intermediate type grew at pH 4.0, where the morphology was smooth and mucoid. In a second experiment, broth cultures of lactobacilli were inoculated in 0.1 ml. amounts into tubes of 0.9 per cent saline at varying pH. At intervals samples were plated, and the number of resulting colonies indicated the resistance of the culture to the pH in question. The data indicated variation in each colony type in response to the different environments.

TABLE IV
ACIDOGENICITY OF LACTOBACILLUS CULTURES IN VARIOUS MEDIA

SOURCE AND TYPE OF ORGANISMS	MEDIUM			
	TOMATO JUICE AGAR, pH 7, 72 HR.	TGE AGAR, 72 HR.	TOMATO JUICE BROTH, pH 7, 48 HR.	HEART INFUSION BROTH, 48 HR.
Immune:				
Smooth	4+	3+	3+	2+
Smooth	4+	2+	4+	2+
Smooth	4+	2+	4+	4+
Intermediate	4+	+	3+	3+
Rough	3+	3+	3+	no growth
Rough	2+	2+	4+	no growth
Rough	3+	±	2+	no growth
Susceptible:				
Smooth	4+	3+	2+	3+
Smooth	3+	±	4+	+
Smooth	4+	3+	4+	4+
Intermediate	4+	−	4+	±
Intermediate	2+	±	4+	−
Rough	−	no growth	±	no growth
Rough	−	no growth	±	no growth
Rough	±	no growth	2+	no growth
Rough	−	no growth	3+	no growth
Rough	−	no growth	4+	no growth
Rough	−	no growth	3+	no growth
Rough	−	no growth	2+	no growth
Rough	−	no growth	3+	no growth

− = No acid produced.
+, 2+, 3+, 4+ = Degree of acid production.

During the studies of lactobacilli on laboratory media, alteration of colony morphology was occasionally seen. Some of these variations were readily reversible by removing the stimulating external condition; others appeared more permanent, although some of the new variants varied again under different conditions. The observations are summarized in Table V. No changes in colony type occurred on solid media at pH 5.0; however, most S type organisms growing at pH 7 on solid media exhibited a form of colony development in which the small first-discernible colony is rough, but with increasing time the colony becomes larger and more smooth until the four-day-old isolated colony is completely smooth. Since at pH 5 even the young colonies are smooth, it would appear that acid produced by the colonies during growth at pH 7 contributes to their becoming smooth.

Variations produced by the S and I types isolated from immune or susceptible saliva were the same. The major difference concerned the R organisms, for while the susceptible R types did not dissociate on the media listed in Table V, the immune R types were capable of doing so frequently both in liquid media and in the presence of tryptone. It is peculiar that the S dissociant resulting from culture on tryptone-containing media was sensitive to the pH of media on which it was grown thereafter, varying from S to R with the change from pH 5 to 7. The S dissociant resulting from repeated culture of these organisms in liquid media was not pH sensitive.

The observation that immune R organisms dissociated to smooth morphology in one passage on tryptone glucose extract agar (TGE) stimulated interest in the component of the medium involved in the variation.

TABLE V
DISSOCIATION OF SEVERAL TYPES OF LACTOBACILLI

SOURCE AND TYPE OF ORGANISMS	ENVIRONMENTAL CONDITIONS AFFECTING VARIATION			
	PASSAGE AT pH 5.0 ON SOLID MEDIA	PASSAGE AT pH 7.0 ON SOLID MEDIA	PASSAGE ON MEDIUM WITH TRYPTONE	PASSAGE IN LIQUID MEDIA
Immune				
S	S	rS	S	rS
S	S	rS	S	rS
I	I	I	I	I
R	R	R	S*	S+
R	R	R	S*	S+
Susceptible				
S	S	rS	S	rS
S	S	rS	S	rS
I	I	I	I	I
I	I	I	I	I
R	R	R	R	R
R	R	R	R	R

* = pH sensitive on solid media: pH 7 = R, pH 5 = S.
+ = Not pH sensitive.
rS = Early colony rough but proceeds to smooth.

Tryptone was the first ingredient of TGE agar to be investigated and it was found that the addition of this to tomato agar caused some R cultures to vary to S morphology. The effect of DL-tryptophane was examined, and it was also found to induce this variation. Demonstration of this phenomenon was not solely dependent on the qualitative presence of the amino acid, but also on the quantity. Further details and studies of this effect of tryptophane have been published elsewhere.[11]

It has been reported that immune saliva is more inhibitory to growth of lactobacilli than susceptible saliva.[12] Measurements of growth of a number of lactobacillus cultures in immune and susceptible saliva, and in mixtures of saliva plus added substrates were made. Freshly isolated cultures of lactobacilli were inoculated from equally turbid saline suspensions into tubes of saliva, saliva + glucose, and saliva + a basal medium, tomato juice broth. The growth in the tubes was determined turbidimetrically, and growth curves of a single typical lactobacillus culture in these substrates are reproduced in Fig. 1. The striking feature observed in these curves is the increase in light transmission occurring in the immune saliva plus basal medium, which is taken to indicate inhibition by immune saliva. This particular pattern was seen only in tubes containing immune saliva and occurred with 9 out of 10 cultures of lactobacilli examined. In every case, the period of increased light transmission, or inhibition, occurred during the first 6 hours after inoculation. The organisms grew in similar manner in these media and could not be distinguished on the basis of source or colony type.

Considering the information shown by these experiments, it may be assumed that immune saliva contains some factor inhibitory to oral lactobacilli, or that it is deficient in some substance necessary for rapid initiation of growth of lactobacilli. With the first assumption, questions arise as to the mode of action of such an inhibitory factor, whether the factor selects out organisms resistant to it thereafter, and whether the factor is exhausted during the process of inhibition.

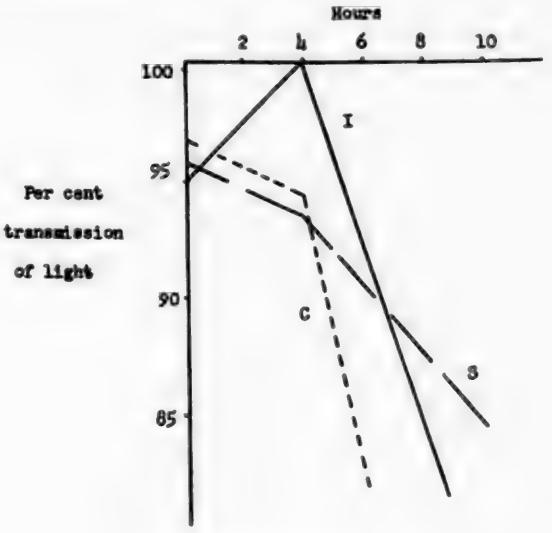

Fig. 1.—Growth curves of a typical lactobacillus culture in immune and susceptible saliva.

Five cultures which had grown for 16 hours in immune saliva + basal medium and in susceptible saliva + basal medium were inoculated into fresh tubes of the same substrates, and these were observed turbidimetrically. No evidence of inhibition occurred in these tubes, seeming to indicate that the organisms were resistant to the supposed inhibitory factor.

Tubes of immune saliva + basal medium in which cultures had grown for 16 hours were rendered bacteria-free by filtration and reinoculated from broth cultures of the same organisms. These tubes were observed and no inhibition of the cultures was evidenced, which might indicate that the supposed inhibitory factor was no longer operating.

Studies have been made of the ability of various oral lactobacilli to grow and produce acid in the presence of saliva, and of the effects of saliva upon the morphology and physiology of the organisms. Filtered pooled saliva obtained from caries-immune and caries-susceptible people was used, undiluted

or with addition of other substrates. As indicated in Table VI there was considerably more acid produced in susceptible saliva than immune saliva, and there appears to be little difference in acidogenicity between fresh and old cultures. The figures S + R in the table indicate a mixture of approximately equal numbers of smooth and rough organisms used in a single inoculum to test the acidogenicity of an association of these types. Another study employed laboratory-maintained strains in substrates consisting of tomato juice broth diluted 1:1 with saline, immune saliva, or susceptible saliva. The pH readings made 10 hours after inoculation with 0.2 ml. of 48-hour broth cultures shown in Table VII again show that while both salivas inhibited acid production, consistently more acid was produced in susceptible compared to immune saliva substrates.

TABLE VI
EFFECT OF AGE ON ACIDOGENICITY OF LACTOBACILLI IN SALIVAS

AGE, SOURCE AND TYPE OF ORGANISMS	IMMUNE SALIVA + GLUCOSE		SUSCEPTIBLE SALIVA + GLUCOSE	
	24 HR.	48 HR.	24 HR.	48 HR.
Fresh, immune				
S_1	–	–	–	–
S_2	–	4+	–	–
R_1	–	–	–	4+
R_2	–	–	–	–
S_1 + R_1	–	–	4+	4+
Old, immune				
S	–	–	–	–
S	–	–	3+	4+
R	–	–	–	–
R	–	–	–	–
Fresh, susceptible				
S_1	–	–	–	4+
S_2	4+	4+	4+	4+
R_1	–	–	4+	4+
R_2	–	–	4+	4+
S_1 + R_1	–	–	4+	4+
Old, susceptible				
S	–	–	4+	4+
S	–	–	–	–
R	–	–	4+	4+
R	–	–	4+	4+

– = No acid produced.
+, 2+, 3+, 4+ = Degree of acid production.
Indicator, Bromcresol purple.

Since immune individuals almost exclusively have smooth lactobacilli in their saliva, the effect of saliva upon colonial morphology has been investigated. Cultures of lactobacilli were inoculated from 18-hour broth cultures into tubes of sterile immune and susceptible saliva. The organisms had been previously maintained through a number of passages on laboratory media. At intervals 0.1 ml. was removed from the tubes, diluted in saline and plated on tomato juice agar at pH 5 and 7. After incubation colonial morphology and numbers were observed on the plates.

Quantitatively, inhibition by immune saliva was seen in several cultures, while in all cases better growth occurred more rapidly in susceptible saliva.

TABLE VII
pH PRODUCED BY LACTOBACILLUS CULTURES IN SALIVA SUBSTRATES AT TEN HOURS

SOURCE AND TYPE OF ORGANISMS	pH		
	TOMATO JUICE + SALINE (CONTROL)	TOMATO JUICE + IMMUNE SALIVA	TOMATO JUICE + SUSCEPTIBLE SALIVA
Immune			
S	4.40	6.86	6.55
R	5.58	6.79	6.50
R	4.76	6.69	6.49
Susceptible			
S	5.20	6.80	6.42
I	6.08	6.75	6.48
R	5.80	6.00	5.61
Original pH	6.70	7.00	6.89

The types of colonial morphology of the organisms appearing on the inoculated plates are summarized in Table VIII. At pH 5, after 24 hours, the original S cultures gave rise to S colonies in both types of saliva. At the same time, 3 out of 4 R organisms had become S in susceptible saliva, and immune saliva had caused only one R organism to dissociate.

TABLE VIII
CHANGES IN COLONIAL MORPHOLOGY OF LACTOBACILLI GROWING IN SALIVAS

SOURCE AND TYPE OF ORGANISMS	GROWTH AT pH 5			
	IMMUNE SALIVA		SUSCEPTIBLE SALIVA	
	6 HR.	24 HR.	6 HR.	24 HR.
Immune				
S_1	S	S	S	S
S_2	S	S	S	S
R_1	R	R	R	R
R_2	S	S	S	S
$S_1 + R_1$	S + R	S + R	S + R	S + R
Susceptible				
S_1	S	S	S	S
S_2	S	S	S	S
R_1	R	R	R	S + R
R_2	R	R	R	S + R
$S_1 + R_1$	S + R	S + R	S + R	S + R
	GROWTH AT pH 7			
Immune				
S_1	R + NS	S + NS	R	R
S_2	R + NS	S + NS	R	S
R_1	R + NS	R + NS	R	R
R_2	R + NS	S + NS	R	S
$S_1 + R_1$	S + R + NS	NS	S + R	S + R
Susceptible				
S_1	S + NS	S + NS	S	S
S_2	R + NS	S + NS	S	S
R_1	R + NS	R + NS	R	S + R
R_2	R + NS	NS	R	S
$S_1 + R_1$	S + R + NS	R + NS	S + R	S + R

NS = New type of smooth colony.
S + R = Mixture of S and R organisms.

Colonies obtained at pH 7 from samples from both kinds of salivas had become rough at 6 hours, in contrast to their appearance at pH 5. This change was also accompanied by the appearance of a new type of colony among organisms plated from immune saliva at 6 hours. This new type, NS, was distinctly different in appearance from any of the other colony types, being flat or convex, round, smooth, and having a blue or bluish-white color. It was often smaller than the other types. The NS type was seen only on media at pH 7 from organisms growing in immune saliva. Usually the NS type increased in numbers to become the predominant colony in 6 to 24 hours, while the number of unchanged colonies remained nearly the same, indicating a selective action by immune saliva. In a few cases, the number of the original type organisms increased considerably, showing that growth of unchanged organisms was not always depressed. All the lactobacilli used in this experiment were able to dissociate to the new type under the influence of immune saliva.

Therefore it appears that susceptible saliva can induce as S to R or R to S variation, as does immune saliva, but the mechanism in immune saliva is different in the S variant which appears. Apparently most of the multiplication occurring in immune saliva was of a nonaciduric form, since much higher counts were obtained at pH 7 than at pH 5.

The capacity of immune saliva to induce the growth of nonaciduric variants of lactobacilli was investigated further using 18 cultures of freshly isolated and older laboratory cultures. Undiluted saliva + 0.5 per cent glucose and saliva + supplement were inoculated with 0.1 ml. of 18-hour broth cultures. The supplement was 1 ml. of a solution of 2 per cent peptonized milk, 0.1 per cent yeast extract, and 1 per cent glucose added to 5 ml. of saliva. No growth or acid production occurred in undiluted immune and susceptible saliva. Acid production in the saliva-glucose tubes has been recorded previously (Table VI). At 48 hours after inoculation, portions of the tubes were plated onto tomato agar at pH 5 and 7. Four cultures from immune saliva showed the NS nonaciduric colonies, which were also seen in 5 cultures from immune saliva + supplement. In addition, 2 other cultures from the latter medium became nonaciduric without developing the peculiar bluish colony. Nonaciduric organisms were not isolated from undiluted saliva, in which none of the organisms grew appreciably, nor from any tubes containing susceptible saliva. Nonaciduric organisms appeared also in colonies which did not resemble the NS type. These will be discussed later.

The acidogenicity of the nonaciduric variants produced by immune saliva was examined, and compared to the acid-producing capacity of the cultures from which they were derived. The results in Table IX show that 5 of the 6 nonaciduric variants recovered after incubation with immune saliva were less acidogenic than the parent cultures. Contact of the cultures with susceptible saliva had little effect on acidogenicity.

These experiments have disclosed a capacity of immune saliva to induce variation of lactobacilli to nonaciduric forms. It was considered possible that

genetic differences might exist in strains of lactobacilli, resulting in varied sensitivity to the transforming effect. In an attempt to determine if most lactobacilli can respond to the effect if proper conditions are met, 18 strains of lactobacilli, some freshly isolated, and some older cultures, were repeatedly transferred at 24-hour intervals in tubes of 3:1 immune saliva and tomato juice broth. At each transfer, a sample of the cultures was plated on tomato juice agar plates of pH 5 and 7, which were observed for appearance of growth. At the end of the first passage, 3 susceptible R cultures, 2 fresh and 1 old, exhibited the nonaciduric NS colonies. At the end of the second passage in immune saliva, all but 5 of the cultures contained a considerable number of nonaciduric forms. These 5 were transferred a third time, after which only 1 failed to show nonaciduric colonies in considerable numbers. The refractory culture was a susceptible intermediate, which had been carried in the laboratory for months.

TABLE IX

ACIDOGENICITY OF IMMUNE-SALIVA-INDUCED VARIANTS OF LACTOBACILLI IN TOMATO JUICE BROTH, pH 7, AT TWENTY-FOUR HOURS

AGE, SOURCE, AND TYPE OF ORGANISMS	ACIDOGENICITY		
	PRIOR TO SALIVA CONTACT	AFTER INCUBATION WITH	
		IMMUNE SALIVA	SUSCEPTIBLE SALIVA
Fresh, immune S	4+	2+	4+
Fresh, immune S	4+	–	4+
Fresh, immune R	4+	4+	4+
Fresh, susceptible R	3+	+	2+
Old, susceptible R	3+	+	3+
Fresh, susceptible R	3+	–	3+

– = No acid produced.
+, 2+, 3+, 4+ = Degree of acid production.
Indicator, Bromcresol purple.

Of the 17 cultures induced to dissociate to a nonaciduric form by passage in immune saliva, 13 produced colonies of the NS type previously described, bluish on the tomato medium, usually small, and smooth. The other 4 produced colonies which were smooth, white, and quite small. The difference in appearance may lie in the ability of the nonaciduric colonies to take up the bromcresol green indicator in the medium. The 4 cultures producing the nonaciduric variants which do not resemble the NS type cannot otherwise be associated on the basis of source, type, or age. However, their nonaciduric property was confirmed by growth in repeated transfers on tomato juice agar at pH 7, but not at pH 5. These variants were shown also to be less acidogenic in tomato juice broth than the original parent cultures.

Turbidimetric studies of growth of lactobacilli in saliva indicated that inhibition by immune saliva occurred during the first 6 hours after inocula-

tion (Fig. 1). The first experiment on the effect of saliva upon colonial morphology disclosed that the nonaciduric organisms were first noticeable at 6 hours (Table VIII). The possibility that these phenomena are related was examined in the following experiment. Organisms were inoculated from 18-hour broth cultures into tubes of 2:1 immune saliva and tomato juice broth. At 2-hour intervals during incubation, samples were taken from the tubes and plated on tomato juice agar at pH 5 and 7. Nonaciduric NS colonies appeared only after the period of inhibition, that is, 6 hours or more after inoculation. Some growth of aciduric organisms occurred after the inhibition period, but the greater amount of growth was of nonaciduric organisms. Thus it appears that inhibition by immune saliva and the ability to induce nonaciduric variants are somehow related.

DISCUSSION

These investigations have disclosed two differences in the salivary microflora of caries immune and caries susceptible human beings, the numbers and colony morphology of lactobacilli present in the saliva. It would appear that some quality in immune saliva is capable of managing the lactobacillus population to reduce their numbers and almost completely abolish rough and intermediate colony morphology in the remaining organisms. Experiments have shown that immune saliva possesses a greater inhibitory capacity for lactobacilli than susceptible saliva. Both types of saliva can induce a rough to smooth variation in colony morphology, but the mechanism in immune saliva is different from the susceptible. It induces nonaciduric variation in lactobacilli, which are then sensitive to acid concentrations around pH 5.

The mechanism in immune saliva is apparently a selection. The data indicate that the processes of inhibition and of selection of nonaciduric organisms are related since evidence of the latter does not appear until the period of inhibition is over. There is some difference in the degree of susceptibility of lactobacilli to this mechanism, which may account for the presence of low numbers of aciduric organisms in some immune salivas.

The effects of immune saliva which have been observed in vitro have acted to alter the cariogenic potential of the lactobacilli by a reduction in numbers and in aciduric and acidogenic properties. If this proposed mechanism operates on the lactobacilli in the mouth, reduction of numbers and activity of these organisms may lower the cariogenic potential of the total acidogenic flora to the point where caries might proceed only slowly if at all. This does not imply that caries without lactobacilli is not possible; it suggests that some reduction of total acidogen activity by inactivation of the lactobacilli may decrease caries rate.

Preliminary experiments in progress on the microflora of plaques of immune and caries susceptible subjects indicate that some of the outstanding features seen in saliva may be duplicated in plaques.

SUMMARY

1. Quantitative comparison of gram-negative diplococci, gram-negative rods, yeasts, caseolytic organisms, streptococci, micrococci, and lactobacilli in caries-immune and caries-susceptible salivas showed that only lactobacilli occurred in significantly different numbers, being much lower in immune salivas.

2. An association of lactobacilli almost exclusively of smooth colony type with immune salivas was found, while susceptible salivas contained more rough than smooth lactobacilli.

3. Dissociation of colony morphology of a number of strains of lactobacilli was found to be influenced by the composition, pH, and physical state of laboratory media.

4. Immune saliva was found to have a greater inhibitory capacity for lactobacilli than susceptible saliva. Both salivas could induce variation of colony morphology of lactobacilli, but immune saliva also induced variation to a nonaciduric form which was less acidogenic than the parent culture.

5. The mechanism in immune saliva is apparently a selection, and is related to the process of inhibition in order of occurrence. The effects of immune saliva could account for the low numbers of lactobacilli compared to susceptibles, and possibly for the low caries rate of immunes by reduction of numbers and cariogenic potential of a part of the acidogenic flora.

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THE FREQUENCIES OF ORAL LACTOBACILLI IN THE HUNT-HOPPERT CARIES-RESISTANT AND CARIES-SUSCEPTIBLE RATS IN RELATION TO THE DEVELOPMENT OF CARIES

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THE etiology of dental caries has been widely investigated in man and animals. Animals have certain advantages over man as experimental subjects and rats have been used to demonstrate that the physical characteristics of the diet,[1, 2] heredity,[3-8] and microorganisms[9] are important factors in the causation of dental caries. It is hoped that further experimentation will disclose the specific organism or organisms responsible for dental caries.

If one uses caries-resistant and caries-susceptible rats, one strain can serve as a control for the other. It has already been demonstrated that lactobacilli are recovered more frequently and in greater numbers from caries-susceptible than from caries-resistant rats.[10-12] However, the prevalence of oral lactobacilli during the life span of these rats has not yet been demonstrated. Since a significant percentage of caries-resistant rats also developed caries, it was decided to determine whether lactobacilli could be recovered as frequently from those animals as from the caries-susceptible rats during the formation of caries. A comparative study was also made of the numbers or oral lactobacilli in both strains of rats.

EXPERIMENTAL METHODS

Rats were examined for oral lactobacilli by brushing their lower right molars with a sterile cotton swab moistened in 1.0 per cent peptone water. For a qualitative test for oral lactobacilli, the swab was retained in 1.0 ml. of 1.0 per cent peptone water. Streak plates on lactobacillus-selection medium (BBL) were made within 1 hour after brushing the teeth. One-quarter of the area of the agar contained in Petri plates was streaked with the swab. Equal numbers of susceptible and resistant animals were tested at the same time. The plates were incubated at 37° C. for 72 hours. An animal was considered to possess lactobacilli when 10 or more colonies were counted in a streaked area. Fewer than 10 colonies was considered inconclusive in establishing the presence of lactobacilli.

For the quantitative determination of oral lactobacilli, the swab was carefully broken off at the tip into 5.0 ml. of 1.0 per cent peptone solution contained in a screw-cap tube. The tube was then thoroughly agitated and dilutions made in distilled water. One-tenth milliliter quantities were plated on the

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surface and the plates incubated at 37° C. for 72 hours. Because of the selectivity of this medium, counts were made if plates had between 3 and 300 colonies.

In all of these experiments the rats were fed the cariogenic diet of Hoppert, Webber, and Canniff.[1, 2] This diet has been modified recently so that only 2 to 3, instead of 70, per cent of the coarsely ground rice is retained on a 20-mesh screen.

RESULTS

A graphic illustration of the frequencies of recovery of oral lactobacilli from caries-resistant and caries-susceptible rats is given in Fig. 1. In this study, the per cent of rats from which lactobacilli were recovered was determined by examining about 25 animals from 4 litters of each strain. The same animals were examined each time, and a total of 29 examinations were made over a 550-day period.

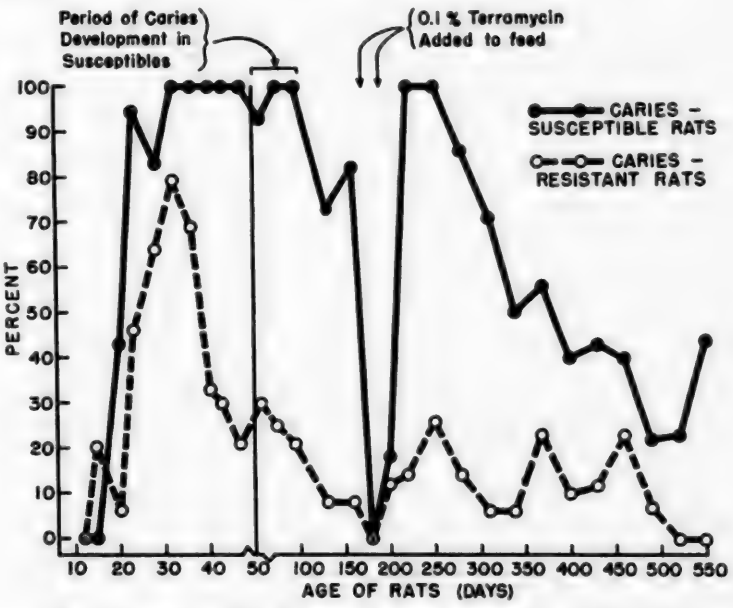

Fig. 1.—Percentage of rats from which lactobacilli were recovered.

Lactobacilli were recovered more frequently from caries-susceptible than from caries-resistant rats. During the early life of both strains of rats there was a parallel increase in the percentages of animals from which lactobacilli were recovered. After the caries-susceptible rats were 21 days old, 100, or nearly 100, per cent recovery was maintained before, during, and after the development of caries. There was a gradual decrease in the per cent of rats from which oral lactobacilli were recovered as they become very old—at an age when the lower central molars and portions of the adjoining teeth had disappeared. This suggests that the surfaces of the teeth, particularly the sulci and the spaces between the teeth, encourage the presence of lactobacilli.

In contrast to the consistent recovery of oral lactobacilli from nearly all of the caries-susceptible rats, there was a recovery in only about 20 per cent of the caries-resistant rats after they were 50 days old. Although there were fluctuations in the recovery of lactobacilli, the incidence remained at a low level for the 550 days the rats were examined. There was no consistent, repeated recovery of lactobacilli from any individual resistant rat on successive examinations. Lactobacilli could not be recovered successively more than twice and in not more than 40 per cent of the total examinations for any single rat after 50 days of age.

During the course of this study, an epizootic threatened the existence of the rat colony and it was necessary to supplement the feed with 0.1 per cent Terramycin for a 21-day period.[13] This resulted in a complete, temporary disappearance of oral lactobacilli.

A second investigation was conducted to determine the difference in numbers of oral lactobacilli between caries-resistant and caries-susceptible rats using lactobacillus-selection medium. The results are given in Table I. These values are the number of organisms per milliliter of the suspension in 5.0 ml. of peptone water.

TABLE I
NUMBERS OF LACTOBACILLI RECOVERED FROM CARIES-RESISTANT AND CARIES-SUSCEPTIBLE RATS

AGE OF RAT (WEEKS)	SUSCEPTIBLE	RESISTANT
6	2,633	476
10	1,252	317
15	2,324	137

Twenty-eight susceptible and 27 resistant rats were used in each of the 3 examinations. Greater numbers of lactobacilli were recovered from caries-susceptible than from caries-resistant rats in each examination. There was an approximate sixfold difference at 6 weeks, a fourfold difference at 10 weeks, and a seventeen fold difference at 15 weeks.

A third investigation compared the percentages of caries-resistant and caries-susceptible rats from which lactobacilli could be recovered during the development of caries. One hundred and twelve resistant and 40 susceptible animals were used in this study. They were examined at approximate 10-day intervals. Susceptible rats were first examined when they were about 40 days old, and the examinations of resistant rats began when they were about 400 days old. Each rat was given between 3 and 10 examinations. The "before caries" period was arbitrarily designated as the time from the first examination to the appearance of the first macroscopic lesion. The results are given in Table II.

Lactobacilli were recovered in 99 ± 0.7 per cent of the examinations of caries-susceptible rats before and after the development of caries.

Lactobacilli were recovered in 36 ± 5.7 per cent of the examinations of resistant rats which subsequently developed caries. This percentage increased to 54 ± 4.6 after the appearance of caries. Among animals which showed no carious lesion, the percentage was very low (13 ± 2.3 per cent).

TABLE II
PERCENTAGES OF EXAMINATIONS OF CARIES-SUSCEPTIBLE AND CARIES-RESISTANT RATS FROM WHICH LACTOBACILLI WERE RECOVERED BEFORE AND AFTER THE DEVELOPMENT OF CARIES

	SUSCEPTIBLE			RESISTANT				
	NO. OF ANIMALS	NO. OF EXAMINATIONS	PER CENT OF EXAMINATIONS FROM WHICH LACTOBACILLI WERE RECOVERED	NO. OF ANIMALS	NO. OF EXAMINATIONS	PER CENT OF EXAMINATIONS FROM WHICH LACTOBACILLI WERE RECOVERED	PERCENTAGE DIFFERENCE	t
Before caries	40	200	99 ± 0.7	31	71	36 ± 5.7	63 ± 5.7	11.1
After caries	40	200	99 ± 0.7	38	120	54 ± 4.6	45 ± 4.6	9.8
No caries	—	—	—	70	207	13 ± 2.3	86 ± 2.4	35.8

To determine what differences between the various groups are statistically significant, t values were calculated. All the differences between the susceptible and resistant groups were highly significant statistically. The 3 t values are 11.1, 9.8, and 35.8. Thus, lactobacilli may be an important cause of the difference in resistance to caries between the susceptible and resistant strains.

There are statistically significant differences between the groups of resistant animals. The difference between the "no caries" resistants and the "before caries" resistants is 23 ± 6.2. The t value is 3.7, which shows that the difference is statistically significant. Thus, the lack of cavities in the "no caries" group suggests that there is a physiologic difference of some sort between these 2 groups. The difference between the "before caries" resistants and the "after caries" resistants, 18 ± 7.3, which gives a t value of 2.5, suggests that the appearance of cavities in the molars of resistants provides a more favorable environment for lactobacilli.

Lactobacilli were found in only 36 per cent of the examinations of resistant rats which subsequently developed caries as compared with 99 per cent for susceptible rats. The difference, 63 ± 5.7, has a t value of 11.1, which is highly significant. It is therefore probable that lactobacilli may cause dental caries in the susceptible animals, but it is doubtful whether they are important etiologic agents of dental caries in resistant rats.

DISCUSSION

The greater frequency of recovery of oral lactobacilli from caries-susceptible than from caries-resistant rats indicates that this group of bacteria is probably involved in the production of dental caries. In caries-susceptible rats, lactobacilli were recovered consistently from almost every rat that was examined between 21 and 250 days of age. Furthermore, lactobacilli were found before as well as after the onset of caries, indicating that they do not appear as a result of caries. They may contribute to the initiation of caries provided that the rats are maintained on a cariogenic diet.

In contrast to the consistent recovery of oral lactobacilli in caries-susceptible rats, only about 20 per cent of the caries-resistant rats yielded lactobacilli after they were 50 days old. There was a higher per cent recovery

from caries-resistant rats when they were younger. A similar reduction has been noted previously,[12] which is an indication that these rats can develop a resistance to this group of bacteria.

When resistant rats developed caries, there was an increase in recovery of lactobacilli when compared with rats of the same strain which did not develop caries. However, the recovery of lactobacilli never approached the frequency found in susceptible animals. Assuming that lactobacilli are the chief etiologic agents of dental caries in the susceptible rats, it appears probable that a different group of organisms is usually responsible for caries in the resistant rats.

SUMMARY

Oral lactobacilli were recovered more frequently and in greater numbers from caries-susceptible than from caries-resistant rats. Lactobacilli were recovered consistently from nearly all of the susceptible rats that were examined. The percentage of resistant animals from which lactobacilli were recovered was high when the rats were very young, but after the animals were 50 days old, lactobacilli were recovered from approximately 20 per cent of the examinations.

Lactobacilli were recovered from resistant rats which subsequently developed caries in only 36 per cent of the examinations as compared to 99 per cent in susceptible animals. This is an indication that lactobacilli may not be important etiologic agents of dental caries in resistant rats, whereas they may be the most important etiologic agent in susceptibles.

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PRECISION OF SALIVARY SEDIMENT TECHNIC FOR EVALUATING INHIBITORS

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A METHOD has been described for study of acid production by a thin layer of salivary sediment coated on a glass electrode.[1] The electrode is immersed in weak bicarbonate buffer containing glucose. Acid formed from glucose within the sediment causes the attainment of a steady state within a few minutes, at which time the pH differential between the sediment and the solution is a measure of the rate of acid production. The influence of some chemicals on the pH differential was used to evaluate the inhibitory effect of such chemicals on acid production.[2-4] The purpose of this study was to determine the reproducibility of the technic under different conditions of test, and to report the precision of the method when it was applied to the routine screening of potential inhibitors of acid production. The essential parts of the equipment are 6 glass electrodes connected in pairs with a common calomel electrode and operating through a Beckman Automatic Switch, a Beckman Industrial pH Meter to a Minneapolis-Honeywell 6 place recorder.

The reproducibility of the technic was studied first with substances capable of producing only a transient change in the pH differential. 0.01 molar sodium dihydrogen phosphate was selected for test because of its ability to increase the buffer capacity of the solution. Since the control buffer solution contained 0.01 molar bicarbonate, the essential effect of the phosphate was merely to double the buffer capacity of solution in contact with salivary sediment. One-tenth milliliter of sediment was placed against each of 3 electrodes, which were in operation for 7 hours and in contact with a variety of substances in addition to the phosphate which is reported in Table I. The figures given are the percentages of the control activity, and were obtained by dividing the second pH differential by the control differential. The average effects on different sediments ranged from 40 to 56 per cent. The standard deviation of a single determination amounted to 11.4 per cent which seems sufficiently precise for a biological technic.

The next trial was made with a substance capable of neutralizing acid in a specific manner (Table II). Urea at 0.01 per cent concentration is converted to ammonia by urease within the sediment, which raises the pH by titrating part of the acid that is formed. This concentration of urea reduces sediment differentials by about one-half as long as the solution is in contact with sediment.

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Sediments were obtained from salivas of 6 subjects and 3 aliquots of each sediment were employed. Two tests were run on each aliquot. The results of the first and second tests are shown separately. The average for the first test was 60 per cent for the whole group. For the second test the average was 48 per cent (12 per cent lower). The standard deviation for all tests is nearly 20 per cent, which is higher than desirable because of a high variance that is attributable to 2 subjects, ME and VJ, whose sediment showed a special susceptibility to urea. The average for the remaining 4 sediments was 70 per cent and the individual values are in good agreement. Possibly the 2 sediments contained a higher urease concentration than did the remainder. Comparison between the aliquots of the same sediments exhibited an average standard deviation of 8 per cent.

TABLE I
VARIANCE WITH A BUFFER
(0.01 M NaH_2PO_4)

	SUBJECTS						
	RM	ME	JB	VJ	WC	CW	MEAN
	51	37	53	60	47	54	50
	47	42	62	46	41	34	45
	41	46	77	47	43	65	53
	41	35	32	32	33	63	39
Mean	45	40	56	46	41	54	47
	S. D. = 11.4						

TABLE II
VARIANCE WITH A NEUTRALIZING SUBSTANCE
(Urea, 0.01%)

	RM		ME		JB		VJ		WC		GH	
	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND	1ST	2ND
Sediment	83	62	29	38	63	49	36	13	76	65	68	67
aliquot	51	67	32	21	72	42	46	22	87	77	69	57
	79	74	36	24	77	72	54	27	56	46	67	35
Average	71	68	32	28	71	54	45	21	73	63	68	53
S. D.	14	5	3	7	5	13	8	6	13	13	1	13

	1ST	2ND	ALL TESTS
Mean	60.0	47.7	53.9
S. D.	18.0	20.1	19.9

In the preceding experiment there was no measure of the substantive of residual effect of chemicals, because the 2 substances chosen did not show such action. In studies of oxygen-containing compounds,[4] and nitrogen derivatives,[2] calculations were made of the percentage change between a first and second control in order to disclose residual influences. A test of reproducibility of a residual inhibitor was based on sodium iodoacetate (Table III). In this instance there were 2 inhibitory effects to be observed. The first was the action while the chemical was in contact with the sediment, and the second was the extent of the recovery of the original pH differential after the chemical had been replaced by control solution. Iodoacetate was employed at a concentration of 10 parts per million because this concentration produced an important inhibitory effect. Fifteen milliliters of saliva from 6 individuals was pooled

and centrifuged in the usual manner and 0.1 ml. aliquots were employed. Six tests were run. The acid production rate was reduced to 37 per cent while the iodoacetate was in contact with the sediment and there was hardly any change during the second control period. Activity after treatment averaged 39 per cent, with a standard deviation of a single determination amounting to 4.3 per cent.

TABLE III
VARIANCE WITH AN INHIBITOR
POOLED SEDIMENT TREATED WITH 10 PPM OF SODIUM IODOACETATE
(% of Control Activity)

	CONTACT	RECOVERY
Aliquots of pooled sediment	36	44
	31	32
	42	35
	44	39
	34	41
	35	43
Mean	37.0	39.0
S. D.	4.5	4.3

Another measure of precision would be the duplicability of the method while being used by different operators for routine tests on a variety of chemicals. During a 3-month period in 1953, there were 4 different operators employed part-time in a general screening program. Our policy was to perform duplicate tests on all compounds that showed recoveries of 65 per cent or less, and single tests on those having higher recoveries (lesser activity). From this group of 300 compounds, there were 57 for which recoveries averaged 45 per cent or less. During this period the test was performed on 0.03 ml. of sediment with 5 per cent supernatant liquid from saliva added to the bicarbonate buffer in order to increase the precision of testing. In certain instances the values differed sufficiently to require that a third test be run, but only the first 2 tests were considered for the calculation of precision. Under these conditions the standard deviation of a single determination amounted to ±7.6 per cent from the mean, with the original control differential equivalent to 100 per cent. This result indicates that two-thirds of the duplicates will check within 15 per cent for a large group of substances whose recovery values are 45 per cent or less.

During the past 3 years several hundred compounds have been tested by the sediment procedure, and enough information has been accumulated to allow us to make a comparison between our findings and those obtained by the conventional saliva-glucose method. Of the 381 compounds tested by Fosdick, Calandra, Blackwell, and Burrill,[5] 46 have been tried by us, usually at a greater concentration. A comparison of findings are given in Table IV. The table is divided according to whether the compounds were effective in inhibiting acid production by both methods, by saliva-glucose method only, or by neither method. The ratio of the effective concentrations were calculated for parts A and B of the table. There were 5 substances which were positive by both tests, but the concentrations required to give a positive test by the salivary sediment was from 20 to over 40 times as great as that required for the saliva-glucose

method. For example, alkyldimethyl benzyl ammonium chloride became completely effective at 0.2 per cent concentration on sediment whereas it was completely effective at 0.005 per cent by the saliva test. No ratio can be given for dodecylamine hydrochloride because our tests were carried out at one-tenth saturated solutions and the exact concentration was not known. Complete inhibition was not achieved with 1 per cent sodium N-lauroyl sarcosinate but the compound was inhibitory to a considerable degree, which indicates that the ratio of effective concentrations would be greater than 40. There are 17 compounds which were inhibitory according to the saliva-glucose technic and not inhibitory by the sediment method. The ratios of the effective concentrations ranged from >1 to >100. In part C of the table are 26 substances which were negative according to both tests. Higher concentrations are needed for inhibitory effect on sediment, presumably because some chemicals are unable to diffuse rapidly into the sediment layer under the test conditions or are eliminated by the rinsing with the second control solution.

TABLE IV
COMPARISON OF SALIVA-GLUCOSE AND SALIVARY SEDIMENT RESULT

	RATIO OF EFFECTIVE CONCENTRATIONS
A. *Compounds positive by both tests*	
alkyldimethylbenzylammonium chloride	40
subtilin	20
decylamine HCl	—
dodecylamine	—
sodium N-lauroyl sarcosinate	>40
B. *Positive by saliva-glucose, negative by sediment*	
Aureomycin	>2
bacitracin	>20
cetyltrimethylammonium bromide	>2
Chloromycetin	>2
dihydrostreptomycin	>2
laurylisoquinolinium bromide	>200
2,2′methylenebis-(4-chlorophenol)	—
penicillin G	>2
soya trimethylammonium chloride	>20
Terramycin	>2
cetyldimethylamine . HCl	>20
formaldehyde	>1
heptaldehyde	>100
tallow amine HCl	>1
tetradecyl amine HCl	>1
isobutyraldehyde	>40
sodium dehydroacetate	>20

C. *Compounds negative by both tests*	
acetyl urea	inositol
dl alanine	isoleucine
L arabinose	L leucine
2-chloromethyl-5-hydroxy-1,4-pyrone	DL norleucine
ethanolamine	DL norvaline
ethyl vanillin	pantothenol
ferrous sulfate	dl phenylalanine
furfural semicarbazone	sodium benzoate
glucosamine	sodium bisulfite
DL glyceraldehyde	tannic acid
glycine	taurine
glycyl glycine	threonine
histamine	dl valine

About half of the compounds tested by Fosdick, Calandra, Blackwell and Burrill, showed activity according to the saliva-glucose procedure.[5] The concentrations were 0.05 per cent or more. About 10 per cent of 1,500 compounds tried by us have shown inhibition greater than 65 per cent in tests by the salivary sediment method. The concentrations usually ranged from 0.1 to 1 per cent. The lower incidence of activity and higher concentrations required both reflect the requirements for diffusibility and substantivity.

SUMMARY

A test of reproducibility has been made for the salivary sediment method[1] of measuring the influence of inhibitors on acid formation rates. The standard deviation of a single determination from the mean was found to range from 5 to 20 per cent, depending on the method used in preparing the sediment and the type of chemical being tested. An over-all standard deviation of duplicates from their means was 7 per cent. Higher concentrations of inhibitory agents are required to inhibit acid formation in salivary sediment than are needed to inhibit acid formation in saliva-glucose mixtures.

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ESTABLISHING AN ENVIRONMENT FOR GROWTH OF ACIDURIC BACTERIA IN THE ORAL CAVITY

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THIS study is based on the assumption that the deposition of dental calculus could be controlled by the production of an appropriate amount of acid by aciduric organisms grown in the mouth. Therefore, it was necessary to find first a practical method to increase the number of lactobacilli in the mouth.

Bunting, Nickerson, Hard, and Crowley[1] found that there was close correlation between activity of dental caries and the presence of *B. acidophilus*. They also noted that there were certain individuals who had a distinct resistance to the growth of *B. acidophilus* in the mouth even though they drank acidophilus milk. They[2] therefore accepted this phenomenon as a presumptive index of susceptibility to dental caries. Howitt, Flemming, and Simonton[3] mention that lactobacilli in the human mouth were the only organisms to respond to an increase of the carbohydrate component in the diet. Bunting, Nickerson, Hard, and Crowley[1] also established that following the experimental feeding of a large number of children for a year on a well-balanced diet from which sugar and candy were practically eliminated, the growth of *B. acidophilus* in the mouth was markedly reduced. The modification of oral flora by diet is extremely difficult. Bibby[4] claimed that it was probable that the mouth flora were less profoundly affected by diet than were those of the intestine because gross accumulations of food were in contact with the teeth for only limited periods of time, while, in the intestine, contact was more prolonged.

METHODS

A small plastic plate was applied to a palate in order to prolong contact of food residue with oral tissues. Subsequently the suitability of the space for fermentation thus provided under the plate was determined by means of lactobacilli counts and determination of the local pH. (Fig. 1.)

At first the number of lactobacilli of the mouth was counted on 5 or 6 successive days in order to estimate the individual level of the lactobacillus count. The average of daily fluctuation and standard deviation were computed. Then the plate was inserted in the mouth and lactobacilli were counted throughout these periods. The pH value on the inner surface of the plate was determined colorimetrically each day of the experiment.

Preliminary investigation showed the following method for lactobacillus count as preferable: Sampling was done just before lunch when oral bacteria residing on and between teeth or on other surfaces in the mouth were collected

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in 10 ml. of sterile peptone water used to wash the mouth. This peptone water consisted of 5 Gm. peptone, 10 Gm. glucose, 2.5 Gm. sodium chloride, and 1,000 ml. distilled water. The pH of this solution was adjusted to 5.0 and its surface tension was measured as approximately 60 dyn./cm. by means of Du Nouy's tensiometer. This produced a slimy saliva, and lactobacilli homogeneously suspended in the mixture could survive for a few hours. A thick foamy layer on the sample, which always arose after vigorous mouth washing, was eliminated by dropping octyl alcohol into the sample. A sufficient amount of peptone water was then added in order to bring the volume up to 20 ml. From this, 0.1 ml. was usually diluted further to 10^2 or 10^3. This produced a final dilution that contained approximately 100 colonies per 0.1 ml. which was seeded as homogeneously as possible on a plated medium consisting of 5 Gm. yeast extract (Difco), 10 Gm. Tripeptone (Takeda), 10 Gm. glucose, 2.5 Gm. sodium chloride, 0.1 Gm. sodium azide, 5 c.c. of 0.2 per cent Brom-Thymol-Green and of 0.2 per cent Cresol-Phenol-Red, 30 Gm. agar-agar, 100 c.c. skim milk, 900 c.c. distilled water, and a small amount of alkali for pH adjustment at 6.0. Incubation was carried at 37° C. for 2 days under microaerophilic condition in a candle jar. On this medium lactobacilli produce colonies with yellowish green halos while proteolytic oral anaerobes produce transparent, somewhat red halos.

Fig. 1.—Palatal plate providing fermentation space.

Strean[5] stated that under anaerobic conditions a greater number of colonies are secured. In this study it was found that culturing under anaerobic conditions produced about 5 times more colonies than under aerobic conditions, but it is believed there is no significant difference between the anaerobic and microaerophilic cultures.

Onisi and Kato[6] showed that when *Esch. coli* suspension was diluted with bouillon, an error of plate counting was introduced which originated with distribution of the colonies on a plate, through errors in pipetting and dilution. When determining the lactobacillus count of saliva, however, the error (represented by standard deviation) through successive dilution is negligible, until the number of colonies on a plate approximates 100. In Table I, variability of pipette errors from each advancing dilution was estimated from 16 to 8.14

per cent while the variability obtained from the dilution containing more or less than 20 colonies was 44.8 per cent. In this high dilution the undetermined increase of the average number of colonies was extremely large. In practice, therefore, the plates which contain over 100 colonies should be chosen for counting.

TABLE I
INFLUENCE OF DILUTION TO LACTOBACILLUS COUNT

DILUTION	NUMBER OF COLONIES ON A PLATE	AVERAGE (M)	STANDARD DEVIATION (S)	VARIABILITY (S/M × 100)	SHIFT OF THE AVERAGES
Original	1,368 1,208 1,200 1,061 1,272	1,222	±101.0	± 8.26%	100
×2	620 685 856 853 754	754	±103.0	±13.7%	123
×4	415 347 317 394 362	367	± 38.6	±10.5%	120
×8	220 178 161 176 233	194	± 31.0	±16.0%	127
×16	104 90 116 100 105	103	± 9.39	± 9.10%	135
×100	19 19 14 17 9 26 36	20	± 8.95	±44.8%	164

FINDINGS

Findings in 8 cases from 6 test persons are surveyed in Table II. These individuals did not suffer from any systemic disease or dental disorder during the period of observation. There was no activation of any lesion during this period.

The daily fluctuation of 16 lactobacillus counts during 20 days for 6 persons was presumed to be a normal distribution; consequently, it was possible to determine the upper limit of rejection at the level of significance 0.05 from the average and standard deviation of preliminary counts. If a count rose over this rejection limit, it was judged to be of significant increase. From this standpoint, 7 cases out of 8 showed a significant increase of *lactobacilli*

TABLE II
TRANSIENT INCREASE OF LACTOBACILLI CAUSED BY WEARING OF PLATE

NAME OF TEST PERSON	DATE OF COUNTING	BEFORE WEARING PLATE				WHILE PLATE WAS WORN		AFTER PLATE WAS REMOVED. COUNT ON PLATE MEDIUM
		COUNTING ON PLATE MEDIUM	AVERAGE	STANDARD DEVIATION	UPPER LIMIT OF REJECTION 0.05	COUNT ON PLATE MEDIUM	Ph OF INSIDE OF PLATE	
		×10⁴	×10⁴	×10⁴	×10⁴	×10⁴		×10⁴
M. O.	1	---				230		110
	2	150				---		149
	3	145	150	12.9	189	630		
	4	152				470	5.2	
	5	172						
	6	132						
		×10⁴	×10⁴	×10⁴	×10⁴	×10⁴		×10⁴
M. O.	1	---				238		144
	2	85				382		164
	3	140	148	34.3	253	504	5.2	
	4	180				479	5.2	
	5	174						
	6	160						
		×10⁴	×10⁴	×10⁴	×10⁴	×10⁴		×10⁴
T. K.	1	---				18		3
	2	9				29		5
	3	5	5.8	2.5	14.7	---		
	4	---				---		
	5	6						
	6	3						
		×10³	×10³	×10³	×10³	×10⁴		×10³
T. K.	1	118				---		230
	2	187				46		219
	3	56	120	47.2	252	82	5.2	
	4	97				210	5.2	
	5	87						
	6	176						
		×10³	×10³	×10³	×10³	×10⁴		×10³
H. M.	1	243				---		73
	2	110				140		240
	3	65	129	63.0	309	230	5.2	
	4	164				56	5.2	
	5	58						
	6	134						
		×10³	×10³	×10³	×10³	×10³		×10³
Y. T.	1	---				60		26
	2	72				100		154
	3	54	45	19.4	104	72	7.0	
	4	53				102×10⁴	5.2	
	5	19						
	6	27						
		×10³	×10³	×10³	×10³	×10⁴		×10³
T. T.	1	---				62		90
	2	145				126		11
	3	112	90	34.3	195	80	5.2	
	4	68				162	5.2	
	5	76						
	6	49						
		×10³	×10³	×10³	×10³	×10³		×10³
N. T.	1	---				78		39
	2	69				80		43
	3	48	45	30.6	138	66	7.0	
	4	86				37	7.0	
	5	6						
	6	15						

after the plate had been worn. The most prominent increase did not usually occur the first day, but rather after the second day of the test period, and was accompanied with a dropping of the pH to 5.2 and also with marked food residue with odor but no putrefaction on the inner surface of the plate. Fig. 2 shows representative cases from the data in Table II. One case (H. T.) showed pH 7.0 through the third day, but on the fourth day there was a drop to 5.2 and an increase in the lactobacillus count to about 23 times that of the individual level. In an exceptional case the count remained on the control level after the plate was worn, showing that local acidity under the plate never developed. In this case the plate was loosely prepared and the inner surface was kept clean throughout this period because of the steady sucking by this individual who was of nervous temperament.

After the plate was removed, the count always came back to within the range of the usual daily fluctuation of each individual.

DISCUSSION

Rodriquez[7] thought that the presence of carious lesions is an indication of caries activity, but Kesel[8] defined caries as inactive when no new cavities developed and there was no increase in extent. It is generally believed that caries activity is related to lactobacillus count. In our experiment, however, there was no sign of a sudden decrease of systemic resistance against infections and no visible expansion of the lesions, although there were increases in the lactobacillus count after wearing a special plate. Thus the resultant increase should not be considered as resulting from the concealed activation of the carious lesion but rather as a result of the favorable environment for lactobacilli provided under the plate.

It is generally accepted that lactobacilli have no catalase, nor microaerophilic or anaerobic oxygen requirements,[9] and that a medium of pH 6.0[10] is favorable for their growth. It is further considered that they first utilize carbohydrate from the media. The decrease of the pH value on the inner surface of the plate to 5.2 favors the predominant growth of lactobacilli over other oral flora. Pincus[11] noticed the reducing capacity of saliva. It is presumed, however, that saliva is always affected by the oxygen tension of the atmosphere, being in contact with the mucous membrane of the mouth. Eisenbrandt[12] obtained a rather high oxidation-reduction potential of saliva, but noted that, if saliva were kept in the sample cup beyond 2 minutes, a considerable, but irregular, drift toward a negative potential began. It is likely that the plate would not only serve to retain the food residue but would also serve to keep oxygen away from saliva, and consequently would preserve a suitable atmospheric cultural environment for oral anaerobes.

Further investigations are needed to confirm our observations on the function of the plate in a mouth. Considering caries activity to be indicated by the lactobacillus count or the capacity for acid production by whole saliva, the narrow space between a clasp and tooth surface and the interdental spaces might be considered as equivalent to the environment provided under the plate. This consideration introduces the possibility that the attempt to express caries activity by lactobacillus count would be influenced by several factors inde-

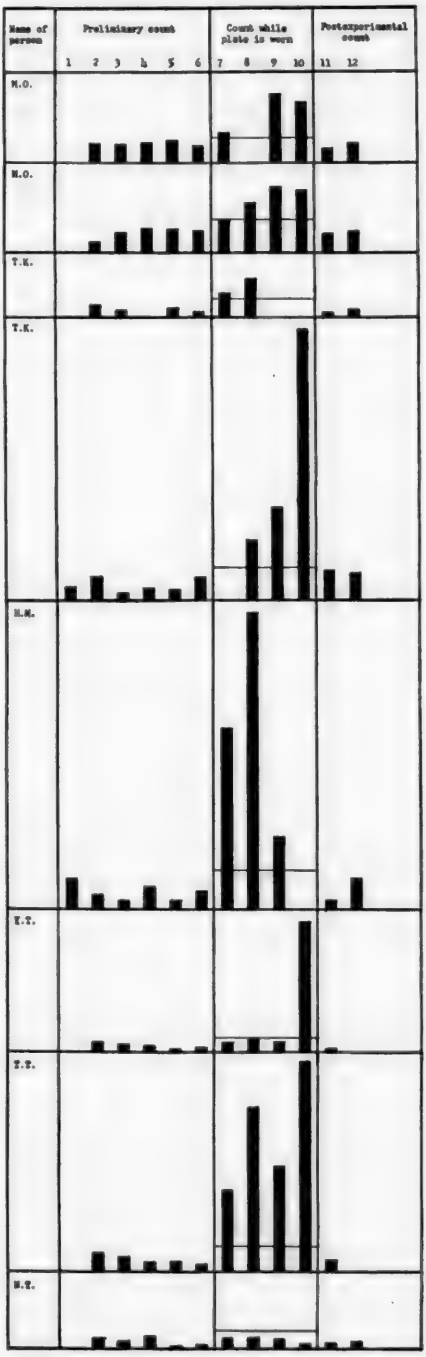

Fig. 2.—Transient change of lactobacillus count by plate placed in mouth. Transverse lines across the columns show upper limits of rejection at the level of significance 0.05. Figures indicate the date of experiment.

pendent of the real cause. For example, Thompson[13] claimed there was difficulty in assaying the possible significance of degree of correlation between caries and the presence of the aciduric bacilli. Tucker[14] could not find persistence of lactobacilli in those cases in which there was shown a high susceptibility to dental caries. Bradel and Blayney[15] observed that in 185 samples obtained during the edentulous period only 20 were positive with very low counts. When a denture was worn, lactobacilli could be recovered consistently, but this finding seemed to be independent of whether the patient was previously classified as caries active or caries free. This clinical investigation is rather understandable from our investigation of the growth environment for lactobacilli provided under the plate.

SUMMARY

Significant sudden increase and return again to the individual level of lactobacilli was observed following insertion and withdrawal, respectively, of a plate applied to the palate. This phenomenon was interpreted as not indicating a fluctuation of caries activity, but resulting from the establishment of an anaerobic fermentation space where lactobacilli could grow predominantly.

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BIOCHEMICAL STUDIES WITH MIXED POPULATIONS OF ORAL BACTERIA

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INTRODUCTION

NUMEROUS quantitative investigations have attempted to determine qualitatively which organisms are the causative agents of dental caries and how enamel is destroyed. Examples of such work are pH measurements of saliva, plaque material, and pure and impure cultures of oral microflora, as well as the classical plate counts for total bacteria, acid formers, and other groups. Much of this voluminous literature has been competently surveyed in 1952 and is now conveniently accessible.[1] However, the constantly changing interrelationships between the mixed microfloral population as a whole and its mean, over-all, or statistical biochemistry have received much less attention. Yet this kind of dynamic approach is suggested by the variability of conditions in the mouth. While some properties (e.g., moisture and temperature) remain fairly constant, others (e.g., diet and pH) vary continuously or discontinuously. These changes may be cyclical (e.g., variation in oxidation-reduction potential) and may result from factors intimately interrelated and exerting a mutual influence upon one another. For example, bacterial action is largely responsible for the pH of the microbic milieu while pH in turn exerts a selective, directive, or otherwise controlling effect on the biochemical activities of the developing culture. Direct qualitative studies of this kind of mass population dynamics would be different from the more static and quantitative technics so far largely employed.

This paper reports experiments on the cultural properties and respiratory activities of mixed populations representing different physiologic groups of oral microorganisms. The objective was more to explore the respirometric methodology for studying the metabolism of oral bacteria in mixed culture than to conduct a comprehensive biochemical investigation. Although the Warburg technic has already been used to measure oxygen consumption by salivary microflora,[2-4] its general usefulness exceeds this particular purpose.

MATERIALS AND METHODS

The composition of the synthetic basal medium employed has been reported.[5, 6] In this mineral solution, a nontoxic nonmetabolizable chelator (ethylenediaminetetraacetate) maintained essential trace metals in a readily available form and enhanced microbial development.[5] To satisfy vitamin requirements[7-9] 0.05 per cent yeast extract was included. Compared with the high substrate levels, this amount of yeast extract would not sustain appreciable

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growth. The complete media (Table I) were designed to simulate carbohydrate, protein, and fat diets, and so obtain in vitro the oral microflora ordinarily developing in vivo under the influence of different diets. In the carbohydrate medium, insoluble starch and powdered $CaCO_3$ were avoided because of difficulty in separation from bacterial cells. The oyster shell fragments neutralized acidity which depressed both growth and respiratory activity. The two lipid media were formulated to obtain different groups of lipolytic bacteria. An organism which utilizes olive oil must possess lipase to hydrolyze the ester, whereas the metabolism of oleate does not require this enzyme. All media were initially adjusted to pH 7.0 and inoculated with about 5.0 ml. of saliva per liter. For aerated cultures 100.0 ml. portions of medium in 250 ml. Erlenmeyer flasks were kept on a continuous horizontal shaker. Stationary cultures consisted of 500 ml. Erlenmeyer flasks filled to the top. These in vitro experiments were attempts to reproduce aerobic and anaerobic or microaerophilic conditions in the mouth. Incubation occurred at 30° C. rather than at 37° C. since the shake machine was in a room-sized incubator set at that temperature.

TABLE I

CONDITIONS FOR CELL PRODUCTION AND WARBURG RESPIROMETRIC EXPERIMENTS

BASAL MEDIUM PLUS (PER CENT)	AGE AT HARVEST, HOURS	PH AT HARVEST	PROPERTIES OF CELL SUSPENSIONS USED: CONCENTRATION FROM ORIGINAL CULTURE*	PREAERATION, HOURS	LUMETRON DENSITY, PER CENT TRANSMISSION WITH 650 RED FILTER†	MG. CELL NITROGEN PER VESSEL (1.9 ML.)	DURATION OF EXPERIMENT, HOURS
			Aerated Cultures				
Glucose 1.0 Sucrose 1.0 Soluble starch 1.0 Oyster shell fragments ca. 25.0	15	7.0	5:1	2 1/3	0	0.79	1¼
Olive oil 1.0	14	6.5	20:1	4½	5	0.82	1¼
Olive oil 1.0 Na oleate 0.2	15	7.4	5:1	2	14	0.86	1½
Gelatin 3.0 Peptone 1.0 Beef extract 0.5 Yeast extract 0.05	19	8.0	2:1	Variable‡	31	0.34	Variable‡
			Stationary Culture				
	15	7.8	20:1	4	39	0.25	1½

*Ratio of $\frac{\text{1000.0 ml. of culture medium}}{\text{ml. of final cell suspension}}$

†Determined after preaeration.

‡Indicated in Table II.

Cultures were harvested by centrifugation for 1 minute at 13,000 r.p.m., washed twice with tap water at 0° C., resuspended in Sørensen's phosphate buffer (M/50, pH 7.0), and refrigerated at 6° C., or frozen at −15° C. (Table II). Cells were starved by aerating the suspensions on the shaker. Manometric experiments were carried out in the Warburg-Barcroft-Haldane respirometer[10] at 30° C., with 16 to 18 ml. flasks containing 0.1 ml. 50.0 per cent KOH in the center well to absorb carbon dioxide and thus permit direct measurement of oxygen uptake. Substrate solutions were prepared with the same buffer to avoid changes in pH and phosphate concentration. The oxygen consumption and quotients[10]

$$[Q_{O_2}\ (N) = \mu l\ O_2 \text{ taken up per hour per milligram of cell nitrogen}]$$

in Tables II, III, and IV have all been corrected by subtracting the corresponding endogenous respiration. These data provide a common denominator for comparing oxidative catabolic activity. Negative values indicate inhibition or suppression of autorespiration.

TABLE II

RESPIRATION OF SUBSTRATES BY ORAL BACTERIA CULTIVATED ON A PROTEIN MEDIUM

SUBSTRATE AND CONCENTRATION PER VESSEL (MG.)	PREAERATION OF CELL SUSPENSION: NONE* — FROZEN 4 DAYS: μL O_2 UPTAKE	FROZEN 4 DAYS: Q_{O_2} (N)	NONE* — UNFROZEN: μL O_2 UPTAKE	UNFROZEN: Q_{O_2} (N)	16 HOURS — UNFROZEN: DURATION OF EXPERIMENT, HOURS	UNFROZEN: μL O_2 UPTAKE	UNFROZEN: Q_{O_2} (N)
Lactate 4.0	109	214	171	405	1.5	120	236
9.1					2.0	186	274
9.1					1.5†	101†	270†
Glucose 4.0	84	165	86	203	1.5	181	356
18.0					2.0	288	423
18.0					1.5†	120†	320†
Sucrose 4.0	65	128	33	78	1.5	30	59
32.0					2.0	70	103
32.0					1.5†	121†	322†
Corn starch 4.0	34	76	−6	−14	1.5	8	16
100.0					2.0	66	97
100.0					1.5†	30†	80†
Lecithin 4.0	25	49	81	190	1.5	64	125
8.0					2.0	166	244
8.0					1.5†	115†	307†
Peptone 1.6 Beef extract 1.6 Yeast extract 0.8	101	198	266	625	1.5	239	468
Gelatin 2.0 Peptone 2.0 Beef extract 2.0 Yeast extract 2.0					2.0	622	915
					1.5†	309†	825†
None (endogenous respiration control)	111	217	126	296	1.5	27	53
					2.0	56	82
					1.5†	20†	53†

*Experiment with unaerated, frozen cells: 1½ hours.
Experiment with unaerated, unfrozen cells: 1¼ hours.
†Cells from stationary (unaerated) culture. All other experiments utilized cells harvested from shake (aerated) cultures.

EXPERIMENTAL RESULTS

Certain observations on the cultures grown for cell production (Table I) give interesting information about the microfloral populations which developed. With carbohydrates, the pH drop in the absence of oyster shell was due at least in part to volatile acids indicated by odor. However, under the experimental conditions employing vigorous aeration, it is highly unlikely that the predominant flora consisted of acidogenic Lactobacteriaceae. Unfortunately, little is known about other oral bacteria that produce acids, which acids they form, in what quantities, and under what conditions. Moreover, acid production in mixed cultures may be quite different both qualitatively and quantitatively than in pure cultures.[11] The calcareous oyster shell which maintained the reaction at pH 7.0 may have neutralized acids that were the same as or different from those produced in the absence of a buffering agent. But it is also possible that less acid may have been formed in the presence of oyster shell. Since pH greatly

influences the elaboration and activity of enzymes, it would be surprising if the biochemical character of the microfloral population did not vary considerably depending upon changes in the reaction of the culture.

The cells grown on gelatin, peptone, meat extract, and yeast extract were characterized by a fecal odor. This was particularly strong in the aerated flasks and differed distinctly from the more wholesome volatile acidic odor of the carbohydrate culture, despite the neutrality of the latter (Table I). The suspension concentrated twentyfold from the 15-hour static protein culture gave a higher density reading but contained less cell nitrogen than the 4-hour older aerated culture concentrated only twofold. The 0.2 variation in final pH of the nonaerated and shake cultures is not enough to account for these differences which indicate that the two cell lots are far from identical. A detailed qualitative comparison of the biochemical nature and especially of the enzymatic armamentaria of the two bacterial populations might be of considerable interest. In general, the degree of aerobiosis of a culture affects the oxidation-reduction potential which, like pH, influences both the production and operation of many enzymes.

TABLE III
OXIDATIVE ABILITY OF ORAL BACTERIA GROWN IN CARBOHYDRATE AND LIPID MEDIA

SUBSTRATE AND CONCENTRATION PER VESSEL (MG.)		CELLS GROWN ON					
		CARBOHYDRATES		OLIVE OIL		OLIVE OIL AND OLEATE	
		μL O_2 UPTAKE	Q_{O_2} (N)	μL O_2 UPTAKE	Q_{O_2} (N)	μL O_2 UPTAKE	Q_{O_2} (N)
Lactate	9.1*	584	591	691	674	122	95
Glucose	18.0*	728	739	784	763	204	156
Sucrose	32.0*	667	676	102	100	17	13
Corn starch	100.0	59	60	44	43	15	12
Lecithin	8.0	482	486	741	722	110	85
Gelatin	2.0	615	622	724	706	503	390
Peptone	2.0						
Beef extract	2.0						
Yeast extract	2.0						
None (endogenous respiration control)		58	58	41	40	219	170

*Equivalent to 100.0 micromoles.

The results with the two fat media also indicate that these cultures are biochemically different. The suspension of cells prepared after 14 hours from the olive oil medium was more than twice as dense but of approximately the same nitrogen content as the 1 hour older olive oil–oleate culture which was only one-fourth as concentrated. The olive oil medium, however, had become acidic (pH 6.5), whereas the reaction in the presence of oleate increased (pH 7.4) due to accumulation of sodium. The significance of this pH difference of almost 1 unit is not known. It may be responsible to some degree for the variation between the cell lots, although the olive oil–oleate medium was generally more nutritious and consequently sustained more luxuriant growth on a cell nitrogen basis. Of the many factors which affect the growth and enzymatic properties of microorganisms, composition of the medium is among the most important.

The oxidative activities of oral microflora selectively grown in media of different composition and the influence of preaeration and freezing are presented in Tables II, III, and IV. The values for the endogenous respiration, corresponding to the basal metabolism of the microflora, indicate the amount of oxidizable reserve food material accumulated during growth. The fact that stored food may or may not be respired simultaneously with externally provided substrates sometimes causes difficulty in interpreting respirometric results. Warburg experiments would give clear-cut data if the endogenous respiration were nil or inappreciable, since all or practically all of the oxygen consumption would then be due to substrate oxidation, but this ideal situation[12] is seldom encountered. Fortunately, however, the endogenous respiration can usually be reduced by preaeration which causes oxidation of reserve food materials.

TABLE IV
COMPARISON OF RESPIRATORY ACTIVITY OF DIFFERENT CELL LOTS*

	Q_{O_2} (N) OF CELLS GROWN ON						
				PROTEINS			
				UNSTARVED CELLS FROM AERATED CULTURE		STARVED, UNFROZEN CELLS†	
SUBSTRATE	CARBOHYDRATES	OLIVE OIL	OLIVE OIL AND OLEATE	FROZEN	UNFROZEN	AERATED CULTURE	STATIONARY CULTURE
Lactate	591	674	95	214	405	274	270
Glucose	739	763	156	165	203	423	320
Sucrose	676	100	13	128	78	103	322
Corn starch	60	43	12	76	−14	97	80
Lecithin	486	722	85	49	190	244	307
Protein	622	706	390	198	625	915	825
None (endogenous respiration control)	58	40	170	217	296	82	53

*Values selected and retabulated from Tables II and III.
†Data for the higher substrate concentrations only.

This effect is shown in Table II where the autorespiration Q_{O_2} (N) of unfrozen protein-grown cells decreased almost sixfold from 296 to 53 as a result of 16 hours preaeration. Rates of respiration of lactate, sucrose, lecithin, and the protein mixture likewise diminished, whereas the corresponding values for glucose and corn starch increased. Such information may be useful in determining whether endogenous respiration continues or abates during oxidation of external substrate and also the degree to which the pathways of metabolism of the reserve food material and external substrate may coincide. In addition, this kind of information may suggest the nature of the end products formed, e.g., acids, alcohols, and so on. While metabolizing their stored reserve food, oral bacteria may produce such substances (including chelators) capable of dissolving apatite in enamel.[6] According to the results in Tables I, II, and III, the autorespiration of cells grown on carbohydrates, Q_{O_2} (N) = 58, and olive oil, Q_{O_2} (N) = 40, was of the same magnitude after preaeration for 2⅓ and 4½ hours, respectively, as the endogenous respiration, Q_{O_2} (N) = 53, of the 16-hour preaerated organisms harvested from the agitated protein medium. On the other hand, the autorespiration of cells harvested from the olive oil–oleate

medium, Q_{O_2} (N) = 170, was 3 to 4 times as great as the corresponding value for cell lots recovered from other aerated cultures. Thus, the bacteria grown on olive oil and oleate appear to have stored considerable food material.

Aside from endogenous respiration, Warburg experiments are sometimes difficult to interpret due to questions concerning permeability of cell membranes. Although readily soluble in water, certain substrates may not penetrate cells due to surface obstruction.[13] This difficulty can sometimes be overcome by freezing, where the formation of intracellular ice crystals ruptures the bacteria.[14] After such disruption, contact between substrate molecules and intracellular enzyme no longer depends upon permeability of the surface membrane. Under these circumstances, the rate of respiration of bulky molecules may then increase, as occurred with sucrose and corn starch (Table II). However, a drastic breakdown of cellular structure may also result in partial or complete loss of enzyme activity, which may account for the depressed Q_{O_2} (N) values of lactate, glucose, lecithin, the endogenous respiration, and especially the combined protein formula (Table II).

Increasing the concentration is another way frequently employed to obtain greater rates of respiration for impermeable or relatively impermeable substrates. In the case of protein-grown cells (Table II), the Q_{O_2} (N) data increased 16, 19, 75, 95, and 506 per cent, respectively, for lactate, glucose, sucrose, lecithin, and corn starch when the higher concentrations were used. With the richer protein formula, the Q_{O_2} (N) value almost doubled. In these studies, regardless of composition of the medium in which the cells had been grown, the addition of glucose invariably caused a marked rise in the rate of gas consumption, whereas a much lesser effect was observed with corn starch. These results, therefore, differ somewhat from observations reported for other studies with glucose and starch,[2, 4] but those Warburg experiments were carried out with saliva, and the concentration of substrates was not the same as in these investigations.

The influence of aeration during growth upon subsequent respiratory activity is shown for starved cells in Table II. With the exception of lecithin and sucrose, the rates of both substrate and endogenous oxidation were higher for cells harvested from shake than from static cultures. With glucose, starch, the protein mixture, and autorespiration, the aerobically grown cells were more active, as one would expect, while the difference of about 1.5 per cent in the case of lactate is not significant. Very likely the Pasteur reaction[15] is somehow concerned with this alteration in oxidative ability depending upon aerobiosis of the growth medium, but the data are too limited to allow serious speculation as to why certain substrates (i.e., sucrose and lecithin) are metabolized more rapidly under aerobic Warburg conditions by cells cultured anaerobically.

A marked difference in the order of substrate oxidation is shown in Table IV. Lecithin, starch, and sucrose were not readily respired by certain cell lots. The variation in general in Q_{O_2} (N) values between the olive oil and olive oil–oleate cells is surprising. The more rapid oxidation of lecithin by cells harvested from the olive oil medium than by those produced in the presence of oleate is what would ordinarly be assumed since both olive oil and lecithin are

glycerol esters. Lecithin is also, incidentally, a phospholipid which may be structurally related to the phospholipid present in the organic matrix of teeth.[16] No cell lots possessed very much amylase activity as indicated by the relative inertness of corn starch. This too is understandable since none of the growth media contained insoluble starch.

The fairly rapid utilization of the protein formula and of lactate by all cell lots can be accounted for on the basis of the tricarboxylic (Krebs's citric acid) cycle which is a major pathway for aerobic terminal respiration.[17] Following hydrolytic degradation of proteins, certain of the resulting amino acids become readily deaminated and otherwise transformed to intermediates of the Krebs cycle.[18] Lactate, on the other hand, needs only to be dehydrogenated to form pyruvate which can then feed into the citric acid mechanism.[19] Lactate was included among the substrates tested because of the prominence accorded this particular anion by the acidogenic theory of dental caries.[20] It is admittedly difficult to demonstrate lactic acid quantitatively in vivo, but some evaluation of the possible role of lactate in dental caries may perhaps be obtained by approaching the problem diametrically; that is, by determining whether this acid, if produced, would persist or be destroyed in the mouth. The results obtained in these investigations suggest that at least under aerobic conditions any lactic acid manufactured in the mouth would probably not be stable for very long because of its susceptibility to microbiological attack. Studies with enzyme poisons[2] indicate that aerobic may be more important than anaerobic metabolism in the mouth. With respect to the acid-decalcification theory, information on the distribution and metabolic activity of oral bacteria that destroy fermentation acids may provide a critical basis for evaluating this concept, and supplement knowledge about the abundance and significance of acid-producing bacteria.[11]

DISCUSSION

These studies were originally undertaken to determine microbiologic stability in the mouth of certain natural-occurring organic compounds with chelating properties.[21] This interest derived from experimental observations suggesting that chelators (also known as metal-binding, sequestering, and coordination complexing agents) may play a role in the formation of dental caries.[6, 22, 23] Growth, per se, was not considered sufficiently sensitive and reliable as a criterion of microbiologic attack since some Protista readily oxidize or otherwise transform substrates on which they are incapable of multiplying. In addition, organisms that have lost the ability to reproduce may still effectuate various enzyme reactions. Consequently, it was decided to measure metabolic activity divorced from cell division, for which the Warburg technic was employed.

The investigations herein reported have attempted to apply some of the research methodologies elucidated by the late Dr. Marjory Stephenson. Her spectrum of levels extending from mixed culture studies in situ to in vitro work with isolated and purified enzyme systems has been presented in some detail by Woods[24] and more recently has been adapted by Hartles[3] to problems of teeth, bacteria, and the oral environment. According to this view, the mouth may be considered a unique microbiologic "niche" in nature with physical, chemical,

and biologic properties distinguishing it from all other locales. But the system is unusually complex to study due to the more or less constant change of many of its major factors. Among these variables, the dietary food intake of the individual is most important. The results of the present studies show, in terms coined by Winogradsky,[25] how zymogenic microflora representing different biochemical and physiologic groups most suited to particular conditions are selected by the available food supply from among the autochthonous (native or indigenous) oral population. Thus, a rapidly changing sequential development of different so-called enrichment cultures occurs, as first appreciated by Beijerinck.[26] The analysis of such a situation may require a kind of "microbiologic calculus" where one variable would be defined in terms of other variables. As reported in this paper, experiments with the Warburg respirometric technic and with mixed groups of oral microflora of different biochemical capabilities may give information on population dynamics of the organisms in the mouth.[27] A similar approach has yielded useful information on certain aspects of soil microbiology, composts, manures, sewage, organic industrial wastes, the rumen, silos, pickling vats, and other mixed populations elsewhere in nature.[28] With respect to the oral microflora, interesting results have already been obtained on the occurrence and activity of several distinct biochemical groups, especially keratinolytic microorganisms which degrade the enamel organic matrix.[6, 29]

SUMMARY

Although the oxygen uptake of saliva has been measured by various technics, including the Warburg respirometer, the advantages of this particular method have apparently not yet been fully exploited for studying the interrelationship between oral microflora and conditions of their environment. The investigations presented here are concerned with the cultural properties and enzymatic behavior of different biochemical groups selected from among the microorganisms of the mouth. The results obtained as a function of differences in composition of the selective growth medium, degree of aerobiosis, pH, and substrate concentration may provide information necessary for understanding the mass population changes which oral microflora constantly undergo. Relatively little has been done so far on the problem of dental caries with this dynamic approach, despite its already established fruitful application to many other phenomena. For these as well as dental caries, the common denominator is a microbiologic milieu in which certain variables constantly change with respect to others.

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LACTIC ACID PRODUCTION IN SALIVA

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ONE of the fundamental premises upon which many current theories as to the mechanisms involved in dental caries are based is the production of lactic acid by bacterial action on carbohydrate food residues in the mouth, which causes acid decalcification of the enamel.[1, 2] Since the production of acid is usually accompanied by a drop in pH, measurements of pH have been widely used to determine acid production.[3] Such measurements are open to the obvious difficulties inherent in well-buffered solutions and do not indicate the amount of acid present. In an attempt to overcome these difficulties others have measured lactic acid production[4] and have used this as a measure of caries susceptibility. In an attempt to assess the validity of such methods the following experiments were performed.

EXPERIMENTAL

The method used to determine lactic acid was the one described by Barker and Summerson,[5] as modified by R. L. Markus.[6]

To see how much lactic acid would be produced by zero-count (lactobacillus index) salivas, 6 such salivas were incubated with buffer and 1 per cent glucose. The mixtures contained 1 ml. saliva, 4 ml. buffer, and 1 ml. 10 per cent glucose and water to 10 ml., and were incubated for 24 hours at 37° C. The results are shown in Table I.

TABLE I

LACTIC ACID PRODUCTION OF ZERO-COUNT (LACTOBACILLUS INDEX) SALIVAS

TUBE NO.	LACTIC ACID PRODUCED AFTER 24 HOURS (MG. PER CENT)
1	45.7
2	18.7
3	14.3
4	16.5
5	41.2
6	44.2

When salivas with variable lactobacillus counts were used, the results obtained are shown in Table II.

These few results indicate that there is no relation between the lactobacillus count of salivas and the milligram per cent of lactic acid produced on incuba-

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TABLE II

RELATION BETWEEN LACTIC ACID PRODUCTION AND LACTOBACILLUS COUNT OF SALIVAS

TUBE NO.	SALIVARY LACTOBACILLUS COUNT	LACTIC ACID (MG. PER CENT)
1	21,000	16.7
2	30,000	61.0
3	60,000	20.6
4	200,000	19.1
5	300,000	45.0

tion. The fact that zero-count salivas produced considerable and variable amounts of lactic acid would indicate that lactic acid production cannot be used as a reliable measurement of lactobacillus concentration. The obvious implication of the results with zero-count salivas is that there are present in the saliva acid-producing bacteria other than lactobacilli.

The pH of saliva when incubated at 37° C. rises from pH 7.0 to 8.0 if no glucose is added. If glucose is added the pH drops, and even quite low concentrations of glucose are sufficient to cause this drop. Wide variations in glucose concentrations above the minimum seem to have little or no effect on the drop in pH or on the amount of lactic acid produced. The total volume in each tube was 10 ml., and 2 ml. of saliva was used in each case.

TABLE III

RELATION BETWEEN GLUCOSE CONCENTRATION, pH, AND LACTIC ACID PRODUCTION

TUBE NO.	GLUCOSE CONC.	pH AFTER 18 HR.	LACTIC ACID AFTER 18 HR. (MG. PER CENT)
1	0.0	8.0	0.0
2	0.1	4.2	39.4
3	0.5	4.6	31.2
4	1.0	4.5	31.8
5	5.0	4.6	40.0
6	10.0	4.5	31.8

TABLE IV

EFFECT OF ADDING UREA TO GLUCOSE-SALIVA MIXTURES ON LACTIC ACID PRODUCTION AND pH

TUBE NO.	SALIVA	GLUCOSE 10 PER CENT (ML.)	UREA 20 PER CENT (ML.)	pH AT END	LACTIC ACID (MG. PER CENT)
1	2	-	1	9.0	None
2	2	-	1	9.0	None
3	2	2	-	3.5	10.0
4	2	2	1	8.8	15.0
5	2	2	1	8.6	12.7

The optimum pH for lactic acid production in the presence of 1 per cent glucose was found to be 6.5.

When urea is added to saliva-glucose mixtures, the pH does not drop, probably due to the fact that the urea in the saliva produces ammonia which neutralizes the acid formed. Strangely enough, the lactic acid production seems

to be stimulated somewhat by the addition of urea. Possibly, the ammonia produced keeps the pH near the optimum for lactic acid production (6.5) for a longer time and thus more lactic acid is produced.

SUMMARY

Lactic acid production in saliva seems to be a poor criterion for lactobacillus concentration since zero-count salivas produce considerable amounts of lactic acid, and in the few cases studied there is no apparent relation between lactobacillus count and lactic acid production.

Quite low concentrations of glucose (0.1 per cent) are sufficient for lactic acid production. The optimum pH for lactic acid production is 6.5. The addition of urea to saliva-glucose mixtures, even though it keeps the pH of the incubation mixture above 7.0, does not inhibit lactic acid production and in this experiment even enhanced somewhat the lactic acid production.

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DECREASED ACTIVITY OF DENTAL CARIES IN DESALIVATED ALBINO RATS

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MOST workers agree that the removal of the major salivary glands results in a pronounced increase in the dental caries experience in the albino rat.[1-4] However, until Hoppert, Webber, and Canniff[5] introduced the coarse corn diet, the albino rat was considered, for all practical purposes, caries resistant. It has been the experience in these laboratories that a high and reproducible incidence of dental caries could not uniformly be produced, even under critical control of such conditions as the source of their diet,[6] their housing conditions, the age of the animal when placed on the cariogenic diet[7] and the type of experimental diet furnished the pregnant animals, young pups, and the experimental animals throughout the course of the test period. Furthermore, the reason for the normally decreased incidence of dental caries in the maxillary arch as compared to the mandibular teeth of both unoperated and desalivated rats, was considered to be of importance in the understanding of the mechanism of dental caries initiation in the albino rat.

Since previous workers have studied the mechanical factors believed necessary for the production of dental caries in both the albino rat[8, 9] and the cotton rat,[10] it was considered of interest to determine if the removal of the major salivary glands in the albino rat would result in the same dental caries experience in the mandibular molars in the absence of the maxillary teeth in comparison to similar animals with the maxillary teeth present. These studies were considered important to determine the relative action of the mechanical factors and of the effect of the absence of saliva on experimental dental caries production in the albino rat, in order that subsequent studies might be directed toward the uniform and reproducible production of dental caries in both the maxillary and mandibular arches. Furthermore, since experimental dental caries studies are so useful for a variety of dental investigations, it would be a welcome adjunct if methods could be devised to produce a high and reproducible caries incidence in the albino rat in both arches in a shorter time interval than is now required.

EXPERIMENTAL

Approximately 100 female weanling rats of the Sprague-Dawley strain were divided into four different experimental groups according to initial body weight. One group was desalivated according to a technic previously described,[4] another was desalivated and, at the same time had all their

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maxillary molars removed with the aid of a No. 701 long shank diamond instrument. The entire clinical crown and root tissue about 1 to 2 mm. below the gingival mucosa was removed. Since the third molars were not completely erupted at this age, particular care had to be exercised to go deeply enough into the gingiva in this area to remove the greater portion of the crown of this tooth. Upon gross examination of the molars at the termination of the experiment, it was found that all of the first and second molars were removed in every animal, but that the clinical crown of the third molars was present to some degree in about 10 per cent of the animals. A third group had only their maxillary molars removed, and a fourth group having neither salivary glands nor molars removed served as controls.

The animals in each of the groups were started on the experiment at 30 days of age. The duration of the experiment was 100 days, after which the animals were sacrificed by ether inhalation and the heads removed for dental caries evaluation by methods previously described.[11] All of the animals received the same stock corn cariogenic diet* (F = 0.5 μg/Gm.) and redistilled water (F = 0.05 μg/ml.), ad libitum. The animals were housed in pairs in raised screen cages in an air-conditioned room.

DATA AND DISCUSSION

The effect on the dental caries experience of the removal of the major salivary glands in albino rats which have their maxillary molars removed is seen in Table I, along with the data from the other three groups. The pronounced increase in the incidence of dental caries in the desalivated rats with maxillary molars confirms much previous work, and is in keeping with the concept that the saliva confers a certain degree of dental caries protection itself, since in its absence dental caries is more rampant. Similarly, it is not unexpected to note that when the maxillary molars were removed the albino

TABLE I

DENTAL CARIES EXPERIENCE (IN THE MANDIBULAR TEETH ONLY) OF ALBINO RATS RECEIVING A STOCK CORN CARIOGENIC DIET AFTER DESALIVATION AND IN THE PRESENCE OR ABSENCE OF THEIR MAXILLARY MOLARS

GROUP	NUMBER OF ANIMALS	WEIGHT GAIN (GM.)	DENTAL CARIES INCIDENCE		
			NUMBER OF LESIONS	M.A.	E.
Desalivate (molars present)	20	113	9.4	5.2	2.5
Desalivate (molars removed)	18	113	1.1	0.9	1.1
Control (molars present)	19	130	4.8	3.3	1.8
Control (molars removed)	21	135	0.1	—	—

*Composition of the diet used in this experiment was as follows: ground whole yellow corn, 12.8 per cent; corn grits, 51.2 per cent; powdered whole milk, 30 per cent; alfalfa meal, 4.8 per cent; irradiated yeast, 0.2 per cent; and sodium chloride, 1.0 per cent. The particle-sized distribution of the total corn components was: 12.4 per cent retained on a 10-mesh screen, 16.4 per cent on a 14-mesh, 50.2 per cent on a 20-mesh, 15.4 per cent on a 28-mesh, and 5.6 per cent passed a 28-mesh screen.

rats had a very low dental caries experience even though they received a corn diet known to induce a moderate caries experience. However, Shaw[12] has reported that albino rats maintained on a carbohydrate-free ration do not develop dental caries even when desalivated. It would appear from these data, that this would indicate quite conclusively that the maxillary molars are necessary for caries development in the albino rat regardless of the presence or absence of saliva, this concept differing from the impression of Van Huysen.[13] The lack of a pronounced effect on the dental caries experience in albino rats, with their maxillary molars removed, weakens somewhat the impression that the salivary gland is of primary importance in inducing dental caries in this rodent. It appears that in order to develop dental caries under these conditions one needs to have an agent that will induce the primary or incipient lesion. If this factor is present, then in the presence or absence of a normal salivary gland function, dental caries will develop, but in the absence of the salivary glands, dental caries will develop at a faster rate. These factors give one the impression that the saliva may be of more importance in the extension of the lesion than in its primary development, and if factors are to be found which will subsequently induce the same degree of dental caries in the maxillary teeth as in the mandibular arch, one must seek agents that will physically impact themselves in the maxillary fissures or adhere to the teeth for prolonged periods of time in order to induce the incipient lesions. The animals in which the salivary glands were removed, in addition to the maxillary molars, had large amounts of debris resembling materia alba present on every surface of the teeth. It was noticeably absent in all other groups, suggesting that it is related to the absence of both the normal cleansing function of saliva and the lack of function that would be furnished if the maxillary molars were present. Even though this debris was at times so extensive as to cover an entire tooth completely, no dental caries developed. The significance of this is not understood.

Close examination of the animals without maxillary molars gave the impression that all the mandibular molars were tipped anteriorly. There may be a relationship not only to the size of the fissure but to their relationship to each other in the respective dental arches and caries resistance or susceptibility. If mechanical factors are primarily responsible for the development of dental caries in the albino rat, a difference in the size of the fissure and position of the maxillary teeth might predispose to caries resistance or susceptibility.

In order to specifically study the effect of the differences in diet on dental caries development in the albino rat, another study was designed in which a high sucrose diet* was used in place of the corn diet. In this experiment one group of animals was desalivated, while the second group was unoperated and served as controls. None of these animals had their molars removed and all received the same diet for 100 days. To further reduce the variable effect of

*The composition of this diet was: sucrose, 55 per cent; vitamin free casein, 30 per cent; inorganic salts, 4 per cent; vitamin mixture, 1 per cent; and Wesson oil, 10 per cent, plus 15 drops of percomorph oil per kilogram of diet.

the diet itself, all of the animals in this experiment were pair-fed by accurately determining the amount of food eaten by a desalivated animal and then providing its paired control the same amount of food. If either of the paired mates failed to eat the entire amount provided during a 24-hour period, the diet remaining was subtracted from the day's total allowance and one gram less was provided for the following day. By increasing the amount of diet by one gram every time the desalivated animal consumed the entire amount, an increased food consumption with increasing body weight of the animal was maintained.

TABLE II

COMPARISON OF DENTAL CARIES EXPERIENCE BETWEEN DESALIVATED ALBINO RATS RECEIVING A HIGH SUCROSE DIET AND AN UNOPERATED CONTROL GROUP RECEIVING THE SAME DIET

GROUP	NUMBER OF ANIMALS	FOOD CONSUMPTION (GM. PER DAY)	DENTAL CARIES EXPERIENCE		
			NUMBER OF LESIONS	M.A.	E.
Desalivated	25	16.4	2.9 (2.4)*	2.7 (2.3)	(1.7) (1.4)
Control	25	16.3	—	—	—

*The numbers in parentheses refer to the dental caries experience when only the mandibular teeth were used for comparison. Note that the small difference is indicative of little caries in the maxillary teeth.

The result of this study is shown in Table II, and indicates that desalivation does not produce as destructive a type of dental caries, even though the maxillary molars are present, when the animals receive a high sucrose diet under pair-fed feeding conditions. These data, like those in Table I, would seem to indicate that desalivation alone is not sufficient to initiate and to cause extension of the carious process in the albino rat in the absence of initiating factors.

SUMMARY

The removal of all maxillary molars prevented the development of dental caries in the mandibular teeth of albino rats receiving a cariogenic stock corn diet, thus indicating the importance of the complete dentition for dental caries initiation and progression. The removal of the major salivary glands did not significantly increase the dental caries experience in the absence of the maxillary molars even though the rats received a cariogenic diet. In desalivated rats which have their maxillary molars present but which are receiving a high sucrose diet, only a small degree of dental caries results. These findings suggest that desalivation per se has little influence on the development of dental caries in the albino rat in the absence of initiating factors.

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A COMPARISON BETWEEN THE WIDTHS OF THE FISSURES OF THE LOWER MOLARS OF CARIES-RESISTANT AND CARIES-SUSCEPTIBLE ALBINO RATS (RATTUS NORVEGICUS)

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THE importance of heredity in the development of dental caries has been demonstrated by Hunt and Hoppert.[4-8] In addition, Hunt, Hoppert, and their associates have shown that the use of the teeth,[11] the coarseness of the rice particles in the diet,[8, 9] and age[9] are also contributing factors. Sex[10] is not a significant factor. Growth rates are not significantly different until the age of 44 weeks.[15] Both male and female susceptible rats have significantly lower hair densities than the resistants.[15] Deviation from the Hoppert diet does not significantly affect the manifestation of hereditary differences between the two strains of rats.[14] Fracturing is not an important factor in the causation of caries in these rats.[13]

Hoppert, Webber, and Canniff,[1] after studying the factor of particle size, reported that lodgment of food particles in the crevices of the teeth appears necessary for the production of dental caries. Other investigators[2] have suggested similar relationships between tooth shape and caries.

Recently, Rosen and his associates have undertaken an intensive investigation of the serum proteins, the properties of the saliva, and the oral flora of the Hunt-Hoppert caries-susceptible and caries-resistant strains of rats. Lactobacilli are found more consistently in the mouths of the susceptible than of the resistant rats,[16] though these may not be the only organisms that are associated with dental caries.[17] Significant differences between caries-susceptible and caries-resistant rats have not been found regarding serum proteins and certain properties of the saliva.[17]

The lower molar teeth of a rat are characterized by deep, narrow, transverse fissures in which food may become impacted. It seems probable that the lodgment of food in such a depression facilitates the carious process. Differences in the widths of fissures might therefore contribute to differences in resistance to caries. The object of this investigation was to determine whether the fissures of the susceptible rats differ significantly in width from the fissures of resistants.

PROCEDURE

Resistant animals used in this experiment were produced from matings of nineteenth generation resistant adults from Hunt's and Hoppert's experiments.

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Susceptible animals used were from matings of twenty-third generation susceptible adults. The animals were fed the H.W.C.[1] cariogenic diet except that 2 to 3, instead of 70 per cent of the ground rice was retained on a 20-mesh screen. The young rats were killed at 40 days of age. The mandibles were removed, numbered, dried, cleaned, and the excess of jawbone was trimmed off. The remaining roughly rectangular pieces of bone, containing the 3 lower molars, were imbedded in dental stone, using the general method of Cox and Dixon.[3] Initial grinding was done on a machine designed by Cox for that purpose.* Final grinding was done by hand.

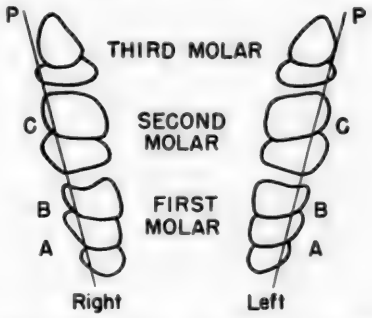

Fig. 1.—Diagram of the lower molars of the albino rat (slightly separated for clarity). *A*, *B*, and *C* are the fissures measured. The approximate plane of measurement is designated by *P*.

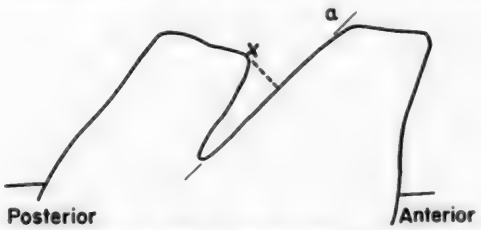

Fig. 2.—Side view of section of the second lower molar. The fissure width is the distance between point x and line a along a line perpendicular to a (dotted line).

When the molars had been ground to the desired level (Fig. 1), they were examined under a low-power microscope. Each of the 3 main fissures (*A*, *B*, *C*, Fig. 1) was measured, using an ocular micrometer (Fig. 2). The fissure width was taken as the distance from the posterior lip of the fissure (x) to the line a, along a line perpendicular to a. Fissures are further identified as being in a lower left molar or a lower right molar. Fissures A and B are in the first lower molar, C is in the second lower molar.

OBSERVATIONS

Both groups of rats (susceptibles and resistants) contained 24 females and 16 males at the beginning of the investigation. Some fissures in the susceptible

*We wish to thank Dr. G. J. Cox of the University of Pittsburgh for the use of his grinding machine and for his very generous instruction in the technic of grinding our specimens.

group were damaged by caries and secondary fracturing following the carious process. Since these fissures could not be accurately measured, they were not included in the study. The numbers of susceptible specimens used were as follows:

	Left Molars	*Right Molars*
Fissure A	20 females – 14 males	19 females – 14 males
Fissure B	20 females – 14 males	18 females – 13 males
Fissure C	14 females – 9 males	14 females – 10 males

In the resistant group the fissures of all the original 24 females and 16 males were measured.

Factors which might, conceivably, affect, or be correlated with, the widths of the fissures in the lower molars are: degree of resistance to dental caries, sex, and the side (right or left) on which the tooth is located.

Any statistical effect of fissure width due to sex and the side on which the tooth is located can be eliminated by comparing the widths of fissure A in the left side of susceptible and resistant males, the widths of fissure A in the left side of susceptible and resistant females, etc., as in Table I. This procedure eliminates from the comparison between resistants and susceptibles the factors of sex and side of the mouth, but it greatly reduces the numbers of animals used in any one comparison. Consequently if differences exist they would not be statistically as significant as when corresponding fissures are compared without regard to sex or jaw.

The widths of fissures in all the tables (except where otherwise stated) are expressed in ocular micrometer units. One ocular micrometer unit was equivalent to 0.195 mm.

All the differences in Table I, except for fissure A on the right jaw among females, have "t" values above 2.0, so that they are statistically significant. Therefore, on the average and in general, the fissures in susceptible animals at the level at which the sections were made are wider than the corresponding fissures of resistant rats.

Table II presents a comparison between susceptible males and females for the same fissure, and between resistant males and females for the same fissure. Sometimes the females have the wider fissure, in other cases the males. The differences are not statistically significant except in the case of fissure B in susceptibles, in which the females appear to have significantly wider fissures than males.

Since sex is, at most, of slight importance, the data for males and females can be combined. This has been done in Table III. The mean width of fissure A on the left jaw of all the susceptibles (males and females) is compared with the mean for A on the left side of all resistants. The same comparison is made for all the fissures on the left and right jaws. In all 6 comparisons the mean fissure width in the susceptibles is larger than the corresponding mean fissure width in resistants. The difference is statistically significant in every case.

TABLE I

COMPARISON BETWEEN FISSURE WIDTHS OF SUSCEPTIBLES AND RESISTANTS

FISSURE	STRAIN AND SEX	AVERAGE WIDTH*	DIFFERENCE	"t" VALUE
		Left Molars		
A	Susceptible males	.70 ± .06	.16 ± .07	2.3
	Resistant males	.54 ± .04		
	Susceptible females	.60 ± .03	.13 ± .04	3.3
	Resistant females	.47 ± .02		
B	Susceptible males	1.22 ± .04	.22 ± .06	3.7
	Resistant males	1.00 ± .04		
	Susceptible females	1.36 ± .05	.31 ± .06	5.2
	Resistant females	1.05 ± .03		
C	Susceptible males	1.37 ± .06	.29 ± .07	4.1
	Resistant males	1.08 ± .03		
	Susceptible females	1.48 ± .07	.41 ± .08	5.1
	Resistant females	1.07 ± .03		
		Right Molars		
A	Susceptible males	.62 ± .05	.16 ± .06	2.7
	Resistant males	.46 ± .04		
	Susceptible females	.63 ± .07	.13 ± .08	1.6
	Resistant females	.50 ± .03		
B	Susceptible males	1.22 ± .05	.16 ± .06	2.7
	Resistant males	1.06 ± .03		
	Susceptible females	1.37 ± .04	.26 ± .05	5.2
	Resistant females	1.11 ± .03		
C	Susceptible males	1.48 ± .05	.45 ± .06	7.5
	Resistant males	1.03 ± .03		
	Susceptible females	1.37 ± .05	.30 ± .06	5.0
	Resistant females	1.07 ± .03		

*In ocular micrometer units. One unit = 0.195 mm.

The incidence of carious cavities is higher in the right lower molars than in the left in both the susceptible and resistant lines,[12] but there are no corresponding differences in the widths of right and left fissures (Table IV). The widths of fissure A on the left and right sides of susceptible males are compared; the widths of fissure A on the left and right sides of susceptible females are compared, and so on for all the fissures in susceptible and resistant animals. All the differences are small; sometimes the left fissure is the wider and sometimes the right, and none of the differences are statistically significant as judged by the "t" value.

Since the differences between the widths of corresponding fissures on the right and left sides are of no significance, one can combine the data for the 2 sides and again compare the mean width of each fissure in males and females. Five of the 6 differences are not significant statistically. But the mean value for the width of fissure B in susceptible females exceeds the mean width in susceptible males by .15 ± .05 units, so that the "t" value was 3.

Fig. 3 summarizes the comparison between fissure widths in the susceptible and resistant rats. For example, the widths of all the A fissures in susceptible

TABLE II

COMPARISON BETWEEN FISSURE WIDTHS IN MALES AND FEMALES

FISSURE	STRAIN AND SEX	AVERAGE WIDTH*	DIFFERENCE	"t" VALUE
		Left Molars		
A	Susceptible males	.70 ± .06	+ .10 ± .07	1.4
	Susceptible females	.60 ± .03		
B	Susceptible males	1.22 ± .04	− .14 ± .06	2.3
	Susceptible females	1.36 ± .05		
C	Susceptible males	1.37 ± .06	− .11 ± .09	1.2
	Susceptible females	1.48 ± .07		
A	Resistant males	.54 ± .04	+ .07 ± .04	1.7
	Resistant females	.47 ± .02		
B	Resistant males	1.00 ± .04	− .05 ± .05	1.0
	Resistant females	1.05 ± .03		
C	Resistant males	1.08 ± .03	+ .01 ± .04	.25
	Resistant females	1.07 ± .03		
		Right Molars		
A	Susceptible males	.62 ± .05	− .01 ± .09	.11
	Susceptible females	.63 ± .07		
B	Susceptible males	1.22 ± .05	− .15 ± .06	2.5
	Susceptible females	1.37 ± .04		
C	Susceptible males	1.48 ± .05	+ .11 ± .07	1.6
	Susceptible females	1.37 ± .05		
A	Resistant males	.46 ± .04	− .04 ± .05	.8
	Resistant females	.50 ± .03		
B	Resistant males	1.06 ± .03	− .05 ± .04	1.25
	Resistant females	1.11 ± .03		
C	Resistant males	1.03 ± .03	− .04 ± .04	1.0
	Resistant females	1.07 ± .03		

*In ocular micrometer units. One unit = 0.195 mm.

TABLE III

COMPARISON BETWEEN FISSURE WIDTHS OF SUSCEPTIBLES AND RESISTANTS

FISSURE	STRAIN	AVERAGE WIDTH	DIFFERENCE	"t" VALUE
		Left Molars		
A	Susceptible	.64 ± .03	+ .15 ± .04	3.8
	Resistant	.49 ± .02		
B	Susceptible	1.33 ± .04	+ .30 ± .04	7.5
	Resistant	1.03 ± .02		
C	Susceptible	1.42 ± .05	+ .35 ± .05	7.0
	Resistant	1.07 ± .02		
		Right Molars		
A	Susceptible	.62 ± .04	+ .14 ± .04	3.5
	Resistant	.48 ± .02		
B	Susceptible	1.33 ± .04	+ .24 ± .04	6.0
	Resistant	1.09 ± .02		
C	Susceptible	1.42 ± .04	+ .37 ± .04	9.3
	Resistant	1.05 ± .02		

rats (in males and females, on the right side and on the left) have been combined in plotting the dotted line for "fissure A." The same combination of data has been made in each of the remaining 5 curves. This procedure is legitimate for comparative purposes because it has been shown that sex and the side of the jaw on which the molar tooth occurs are of little importance in determining the widths of the fissures. It has been mentioned that the width of fissure B is probably, on the average, significantly wider in susceptible females than in susceptible males. This appears to be characteristic of the caries-susceptible rats, and therefore enters legitimately into the comparison

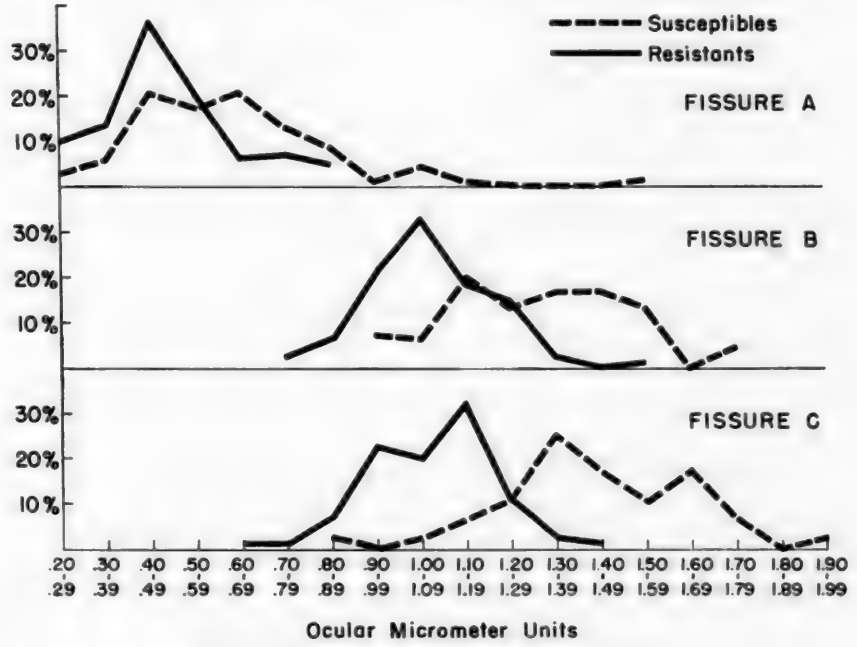

Fig. 3.—Percentage distribution of fissure widths.

between susceptibles and resistants. The greater average width of fissure B in susceptibles, as compared with resistants, is not due to a larger proportion of females in the susceptible sample, because 58 per cent of this sample consisted of females, and 60 per cent of the resistant sample used for fissure B were females.

Fig. 3 convincingly confirms the view that, on the average, the fissures measured in the lower molars of our inbred caries-susceptible rats are wider than the corresponding fissures in the inbred resistants. Measurements were made on the fissures in the lower molars of a few rats used in Nakfoor, Hunt, and Hoppert's[13] investigation, and the same fact was observed. Hoppert, who examines all of our living rats to identify carious cavities, has frequently remarked that susceptible teeth appear to have wider fissures than the resistants.

It is equally clear that the difference in fissure width is not the only cause of the difference in susceptibility to dental caries shown by the 2 strains, for the curves overlap extensively. Many susceptible animals have fissures as narrow as the resistants.

TABLE IV

COMPARISON BETWEEN FISSURE WIDTHS OF THE RIGHT AND LEFT SIDES

FISSURE	STRAIN, SEX, AND MANDIBLE	AVERAGE WIDTH	DIFFERENCE	"t" VALUE
A	Susceptible males, left	.70 ± .06	+ .08 ± .08	1.0
	Susceptible males, right	.62 ± .05		
A	Susceptible females, left	.60 ± .03	− .03 ± .08	.4
	Susceptible females, right	.63 ± .07		
B	Susceptible males, left	1.22 ± .04	0 ± .06	.0
	Susceptible males, right	1.22 ± .05		
B	Susceptible females, left	1.36 ± .05	− .01 ± .06	.2
	Susceptible females, right	1.37 ± .04		
C	Susceptible males, left	1.37 ± .06	− .11 ± .08	1.4
	Susceptible males, right	1.48 ± .05		
C	Susceptible females, left	1.48 ± .07	+ .11 ± .09	1.2
	Susceptible females, right	1.37 ± .05		
A	Resistant males, left	.54 ± .04	+ .08 ± .06	1.3
	Resistant males, right	.46 ± .04		
A	Resistant females, left	.47 ± .02	− .03 ± .04	.8
	Resistant females, right	.50 ± .03		
B	Resistant males, left	1.00 ± .04	− .06 ± .05	1.2
	Resistant males, right	1.06 ± .03		
B	Resistant females, left	1.05 ± .03	− .06 ± .04	1.5
	Resistant females, right	1.11 ± .03		
C	Resistant males, left	1.08 ± .03	+ .05 ± .04	1.3
	Resistant males, right	1.03 ± .03		
C	Resistant females, left	1.07 ± .03	0 ± .04	0
	Resistant females, right	1.07 ± .03		

DISCUSSION

Without doubt there was some unavoidable variation in the level at which the measurements were made. However, the buccal crests of the molars were used as markers during both mounting and grinding. During the later stages of the grinding, the plaster was carefully scraped away from the cusps of the teeth. This enabled the experimenter to see the level at which he was working. The variation in the plane of sectioning, therefore, is believed to be approximately the same for both susceptible and resistant groups.

Extensive microscopic caries were observed in the sectioned teeth of the susceptible animals. If caries or other injury to the tooth removed material in the region at which the width of the fissure was measured (Fig. 2), the measurement would be worthless. Of the 228 fissures in the susceptible group, 223 showed caries or decalcification of the enamel. Among the 240 fissures of

the resistant rats, five A and three C fissures showed slight decalcification of the enamel. One doubtful case was found. One instance of secondary caries due to fracturing was identified.

Only those fissures in which there was no damage at point x and in which plane a (Fig. 2), the other limit of measurement, could be located were measured. The discarding of fissures was so critical that some intact ones were probably rejected because there appeared some grounds for suspecting damage to the measured region.

Theoretically, it might be contended that measurements of the fissures should also have been made at several other planes. The plane chosen can be regarded as a random selection of planes. Therefore, further laborious investigation appeared to be unnecessary.

The cavities observed by Hunt and Hoppert are possibly, as a rule, initiated by caries which weaken the tooth, and this is followed by secondary fracturing that makes the cavity visible to the unaided eye. "Caries time," as defined by Hunt and Hoppert, is the difference between 35 days of age and the age at which caries is first observed. The caries time was 23 days for the twenty-fourth susceptible generation, which was the generation used in the measurement of fissure width. Thus, gross carious lesions were observed by Hunt and Hoppert in this generation at the average age of 58 days. Both microscopic caries and secondary fracture due to caries were present at 40 days of age among susceptibles in this experiment. This suggests that the caries time observed by Hunt and Hoppert might actually be the time at which secondary fracturing occurred following caries.

Hunt and Hoppert[12] reported on the distribution of carious cavities in these strains of rats. Table V correlates the locations of carious cavities with the widths of fissures in the lower molar teeth. It is evident that width of fissures is at most only one factor in the causation of caries. Only 39.5 per cent of the carious cavities on the right side of susceptibles and 20.9 per cent of the caries on the left side of susceptibles were found at fissures. Corresponding frequencies for resistant rats were 11.4 per cent and 4.2 per cent.

TABLE V

CORRELATION BETWEEN FISSURE WIDTHS AND FREQUENCIES OF CARIES

KIFER-HUNT DESIGNATION	HUNT-HOPPERT DESIGNATION	SUSCEPTIBLE SERIES		RESISTANT SERIES	
		FISSURE WIDTH (MM.)	INCIDENCE OF CARIES	FISSURE WIDTH (MM.)	INCIDENCE OF CARIES
Right A	Right A	.12	0	.09	.03%
Right B	Right B	.26	16.4%	.21	4.4%
Right C	Right F	.28	23.1% } 39.5%	.21	7.0% } 11.4%
Left A	Left A	.13	0	.10	0%
Left B	Left B	.26	5.3%	.20	1.4%
Left C	Left F	.28	15.6% } 20.9%	.21	2.8% } 4.2%

Hunt and Hoppert[12] did not find carious lesions at fissure A in susceptible rats. However, examination of the ground sections revealed caries with equal frequencies at all fissures of the susceptible rats. The depths of the fissures

suggest an explanation. Fissures B and C are deeper than fissure A. All the fissures are inclined forward. Possibly the longer, unsupported posterior walls of fissures B and C are more liable to fracture after undermining by caries than the posterior wall of fissure A. Such secondary fracturing would result in a gross lesion, easily seen in macroscopic examination.

Do the different average fissure widths of the susceptible and resistant strains of rats contribute to the difference in mean caries times in these two strains? It is conceivable that the association between the wider fissures of the susceptible rats with the relatively short time required to develop dental caries is fortuitous, that is, that the two traits have been fixed in the strain by inbreeding and have no causal relation. But dental caries is promoted by the presence of coarse rice particles in the H.W.C. diet, and impaction of food is known to occur in the fissures of the molar teeth of rats. The writers are inclined to favor the hypothesis that the wider fissures facilitate the impaction of food containing the coarse rice particles, and that these impactions favor the growth of cariogenic bacteria in the fissure. Where wide fissures occur in the molars of resistant animals, caries may not develop because another inherited condition in the mouth prevents caries in spite of the width of the fissure. On the other hand, an inherited factor might cause caries in the mouth of a susceptible rat in spite of a narrow fissure. It is, of course, conceivable that wider fissures provide greater opportunities for an impaction to be washed out by the saliva. It is clear that width of fissure can only be one of perhaps many factors which affect dental caries. An investigation of the relation between fissure width and caries time within the susceptible line is desirable.

SUMMARY

1. The fissures in the left and right lower molar teeth of caries-susceptible rats are, at the plane studied, significantly wider than the corresponding fissures in caries-resistant rats at the age of 40 days.

2. Fissure width may be one of the factors involved in the etiology of dental caries in rats.

3. The presence of extensive microscopic caries in 40-day-old caries-susceptible rats is discussed.

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PREVALENCE OF DENTAL CARIES IN YOUNG ADULT MALES IN ISRAEL (18 TO 30 YEARS OF AGE)

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INTRODUCTION

THE purpose of this investigation was to determine the prevalence of dental caries in the adult male army personnel (18 to 30 years of age) in Israel; to compare the incidence of caries in the maxilla and mandible; and to establish the incidence and number of affected first permanent molars. The purpose of analyzing this last factor, the incidence of first molar caries, was to test the validity of using the first permanent molars as a measure of total caries experience in epidemiologic surveys at the ages of 18 to 30 years.

REVIEW OF LITERATURE

The simplest method of surveying the occurrence of caries epidemiologically is to determine the proportion of the population affected by it (per cent of persons affected). Kiernan[6] used this method as early as 1892, and it has since been followed by many others.[1, 7, 13]

This method does not indicate, of course, the amount of caries present, since a person with a single carious lesion receives the same statistical weight as one with rampant decay. Therefore a logical method of quantitatively evaluating the prevalence of caries is to count the number of teeth per person which are affected by dental decay. This approach found its culmination in the method of counting decayed (D), missing (M), and filled (F) teeth.[7] As in any other disease, the total caries experience can be obtained by adding the amount of morbidity (number of decay and filled teeth) plus the mortality (number of extracted teeth).

It is well established that the first permanent molars are most frequently and severely affected by caries.[1, 7, 11, 14] Cheyne and Drain[1] showed that the first permanent molars contributed 88 per cent to the total caries experience while the 2 lower first permanent molars alone contributed 65.8 per cent to the total number of DMF teeth at early ages. On this basis, Knutson and Klein[16] suggested the use of the first permanent molars as a measure of caries experience. Massler and Chand[9] showed the validity of using this method up to 12 years of age.

MATERIALS

This study is based upon the oral examination of 7,727 young male adults, 18 to 30 years of age (Table I). The young men were a fair sample of the

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TABLE I

CARIES EXPERIENCE IN 7,727 YOUNG MALE ADULTS OF ISRAEL (18-30 YEARS OF AGE)

AGE IN YEARS	NUMBER OF MEN EXAMINED	PER CENT OF PERSONS AFFECTED BY CARIES	PER CENT OF PERSONS WITH AFFECTED FIRST PERMANENT MOLARS	AVERAGE NUMBER DMF TEETH PER PERSON			AVERAGE NUMBER DMF FIRST PERMANENT MOLARS PER PERSON		
				UPPER	LOWER	TOTAL	UPPER	LOWER	TOTAL
18	548	81.39	76.10	2.107	1.550	3.657	0.879	1.104	1.983
19	1036	86.59	77.80	2.056	2.035	4.091	0.930	1.104	2.034
20	1055	86.07	76.30	2.326	1.927	4.253	0.961	1.136	2.097
21	802	88.53	79.68	2.567	2.015	4.582	0.963	1.234	2.197
22	677	91.73	85.23	2.598	1.698	5.296	1.082	1.305	2.387
23	612	91.02	89.55	3.637	2.338	5.975	1.173	1.344	2.517
24	666	93.25	81.34	3.489	2.571	6.060	1.133	1.339	2.472
25	634	93.54	86.44	3.659	2.561	6.220	1.187	1.343	2.530
26	639	95.94	90.15	4.097	2.621	6.718	1.250	1.463	2.593
27	517	95.75	88.59	4.125	2.847	6.972	1.214	1.429	2.643
28	249	95.59	91.17	4.373	2.775	7.148	1.389	1.485	2.874
29	167	98.81	92.82	4.586	3.054	7.640	1.401	1.514	2.915
30	125	96.80	92 80	5.264	3.360	8.629	1.376	1.648	3.024

present population of Israel, and included, in addition to those born in Israel, immigrants from different parts of the world. All the men were in good health and had been declared fit for military service. Since no dental cause exists for inadmittance to the service, this study reflects the dental conditions of all individuals who met the general medical standards for admission to the military.

Since their period of service in the armed forces was relatively short, the army diet had no influence on the quality of their teeth. Before their induction they all adhered to the diet customary in their respective communities. These varied greatly from typically Eastern to typically Western dietaries.

While all men received adequate dental care during their army service, most of the immigrants from Europe had lived under extremely hazardous conditions during the war years and presented with rather neglected dentitions.

METHODS

Examination.—The oral examinations were made with No. 4 magnifying mirrors and fine, pointed pig-tail explorers under good natural lighting conditions. A chip-blower, cotton pellets, and cotton tweezers were available to aid in the diagnosis of doubtful cases. The lesions recorded were those found readily on careful, clinical dental examination.

Pits and fissures were thoroughly explored and not counted as a carious defect unless there was visual evidence of caries, or if the explorer actually penetrated into soft dentin. A tooth that had been previously filled and had developed secondary caries since, was counted only once as having active decay.

FINDINGS

Per cent of Persons Affected by Caries.—

DMF teeth: Table I and Fig. 1 indicate the per cent of men affected by caries. At the age of 18 years 81.39 per cent were already affected by caries. This attack rate showed a rapid acceleration so that at 19 years 86.59 per cent were affected. The curve in Fig. 1 shows a slight drop at the age of 20 at which 86.07 per cent were affected. This and similar drops in the number of persons affected are undoubtedly sampling errors caused by the fact that this is a cross-sectional study recording the caries experience in different persons at different ages, rather than a longitudinal study of findings in the same people at different ages. Since caries is a cumulative disease and evidence of its existence is never obliterated from the mouth, a drop in the number of carious teeth with advancing age is an artifact. From 20 to 28 years of age, a steady but slower increase in the percentage of people affected by caries can be noted in Fig. 1. The curve fitted to the plotted data by the method of least squares shows the trend of increased caries experience with advancing age.

DMF first permanent molars: Table I and Fig. 1 show the percentage of persons with first permanent molars attacked by caries. At 18 years of age 76.10 per cent of the soldiers examined had first permanent molars affected. Thereafter, the increase in the incidence of caries in the first permanent molars was steady with only slight drops at the ages of 20, 23, and 26 years. As explained previously, these fluctuations are sampling errors, variables inherent in

cross-sectional data using only moderate-sized samples. The interpolated curve shows the trend of increased damage to the first permanent molars with advancing age.

Average Number of DMF Teeth Per Person.—

Table I and Fig. 2 indicate that the number of DMF teeth at the age of 18 was 3.66 per person. From 19 to 30 years of age this figure showed a steady rise, so that at 30 years of age the average number of DMF teeth per person was 8.62. The curve fitted to the data by the method of least squares shows the trend in caries experience to increase approximately in a straight line.

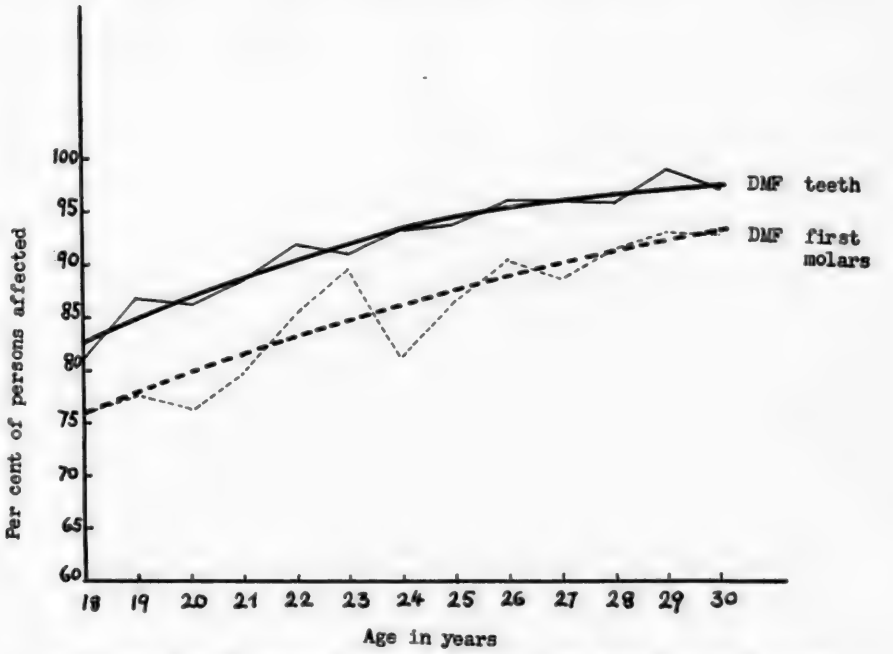

Fig. 1.—Per cent of persons with DMF teeth and DMF first permanent molars.

Difference between maxillary and mandibular arches: Fig. 2 also indicates the number of DMF teeth in the maxillary and mandibular arches as compared to the total. Both curves show, with slight exceptions, a steady increase in the number of DMF teeth with the increase in age. However, the increase in caries experience is faster in the maxilla than in the mandible so that an appreciable difference exists between the two jaws at 30 years of age.

Average number of carious (DMF) first permanent molars per person: Table I and Fig. 3 show the caries experience in the first permanent molars from the ages of 18 to 30 years. The total number of DMF first permanent molars at 18 years was almost 2 (1.983) per person. Thereafter the number of DMF permanent first molars increased steadily (with some slight fluctuations) and reached 3,024 at the age of 30.

Fig. 1 compares the DMF rate in the maxillary and mandibular first permanent molars. In contrast to the curves for the entire maxillary and mandibular arches (Fig. 2), the mandibular first molars were more often affected than the maxillary.

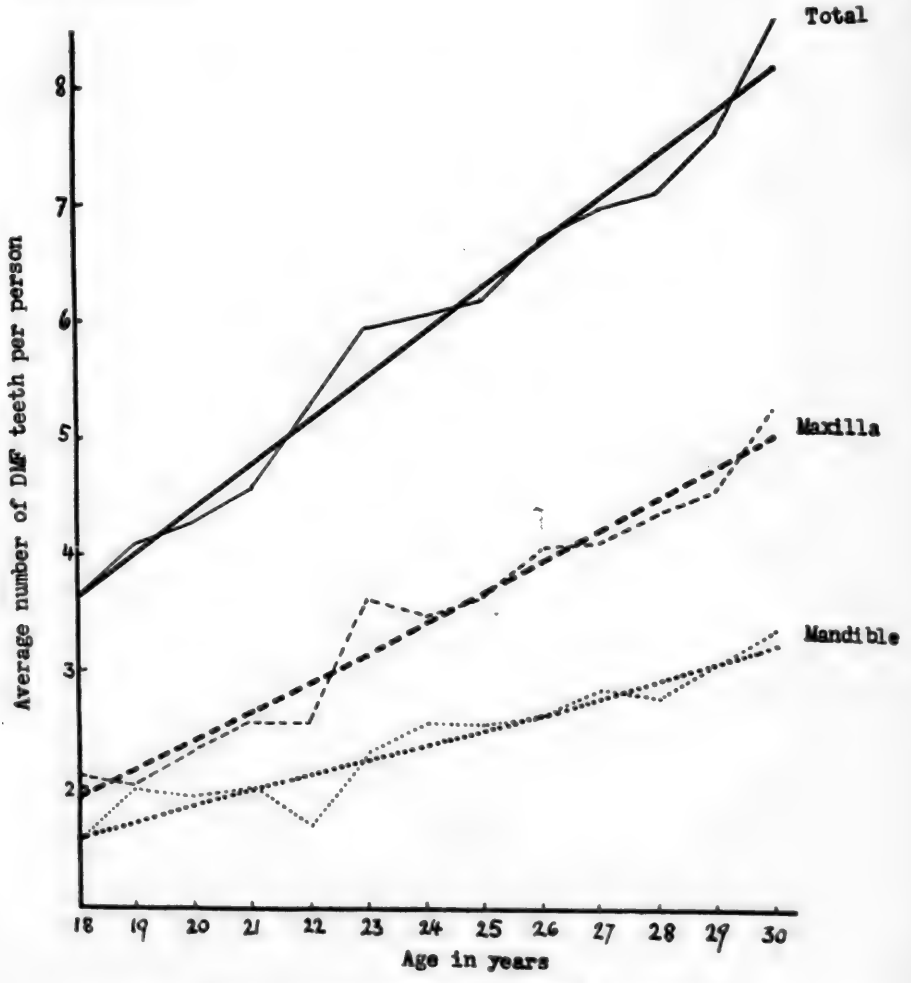

Fig. 2.—Average number of DMF teeth per person.

Comparison of Caries Experience in All Permanent Teeth With Caries.—Experience in first permanent molars: Since one of the objectives of this study was to analyze the validity of using the first permanent molars as an index of the total caries experience in all permanent teeth at an adult age (18 to 30 years), the caries experience in all permanent teeth was compared with that in the first permanent molars.

A coefficient of correlation was calculated for the number of DMF teeth and number of DMF first molars. This coefficient was +0.317 and thus showed that between the ages of 18 and 30 years the first molars were no longer representative of the caries experience in all the permanent teeth.

Differences in Caries Experience According to Country of Origin.—Very striking differences were noticed in the prevalence of caries in men who were born in Israel and countries of the Middle East, and those who came from Europe and America. Those born in the Middle East were strikingly caries immune. Unfortunately, however, data as to ethnic origin were not collected in all examinations and therefore no detailed analysis can be made at this point.

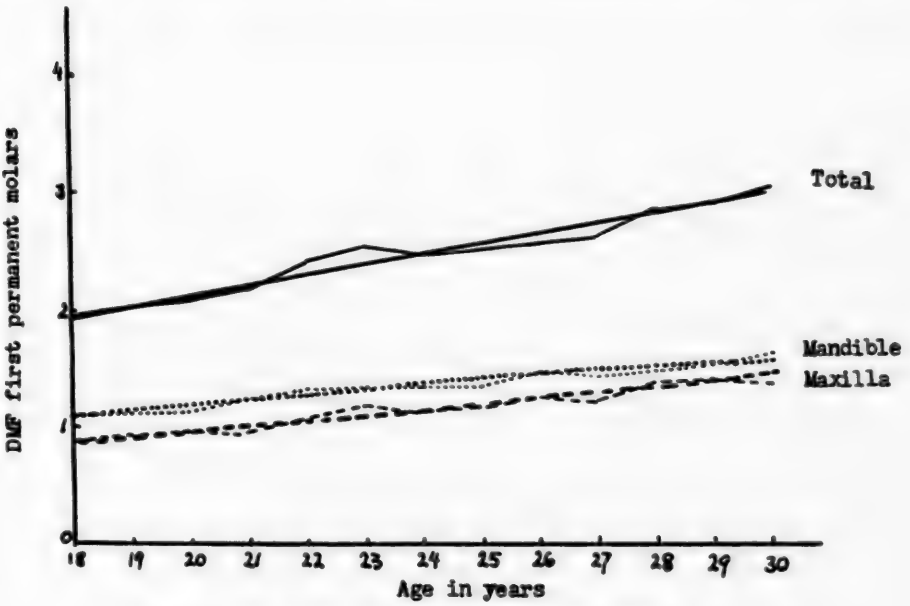

Fig. 3.—Average number of DMF first permanent molars per person.

DISCUSSION

DMF teeth: The average number of DMF teeth per person examined in Israel appears to be much lower than those reported for similar age groups elsewhere. Hollander and Dunning[17] examined 8,410 office employees of an insurance company in New York City and found 15.82 DMF teeth at ages 20 to 24 and 18.28 DMF teeth at the ages 25 to 29 years. This is in contrast to the findings in Israel of 5.23 and 6.94 respectively.

Although there is considerable evidence to indicate that the DMF figure may vary considerably depending upon the experience and care exercised by the examiner,[3] it is felt that these variations are not sufficient to account for the striking difference in the caries rate. A significant number of the men ex-

amined were born in Israel or Middle East countries. These men were strikingly caries immune. The low caries figure is undoubtedly related to this fact.

The reason for the relative caries immunity in those men from the Middle East is at this time speculative. Though a high amount of sunshine, as prevails in these parts of the world, was suggested as cause by Mills[10] and by East,[4] this finds no support epidemiologically when analyzed in the light of present knowledge. Another factor which might account for the low caries experience in those from the Middle East is the chemical composition of waters consumed as suggested by Mills[10] and others.[2, 5] Klein and Palmer[8] have shown that the hardness of the drinking water and caries prevalence are significantly and inversely related to each other. Since the total hardness of the drinking water in Israel is extremely high, this might be a contributing factor to the very low caries experience of the men born in Israel. However, when we relate dental caries experience to domestic water on the basis of its hardness, we are very apt to be relating it also to a higher fluoride content unknowingly.[15] The water sources of this country—such as have been examined so far—show a sodium fluoride content of from 0.3 to 1.3 parts per million. If we take into account the bigger quantities of water consumed in this hot climate, it is possible that the amount of fluoride ingested approaches the optimum level. Finally, the pattern of fermentable carbohydrate ingestion in the diet must be thoroughly analyzed before definite conclusions may be drawn.

SUMMARY AND CONCLUSIONS

The caries experience of a group of 7,727 young male soldiers 18 to 30 years of age was assessed by simple clinical examination of the teeth and scoring the DMF rate for the group. The caries experience of the first permanent molars was also calculated in order to test the validity of using the first permanent molar as an index of the total caries experience at this age. The caries rate was computed separately in the upper and lower arches in order to establish the relative susceptibility of each.

The findings were:

1. The number of DMF teeth was rather low when compared with data from other countries.

2. Caries prevalence among those born in Israel and the Middle East was exceedingly low. This portion of the sample accounted for the rather low caries rate for the group.

3. The mandibular first permanent molars were more often affected by caries than were the maxillary ones. There was a relatively low correlation between the caries prevalence in the first permanent molars and total caries experience. Therefore the first permanent molars do not provide a reliable index for total caries experience at the ages of 18 to 30 years.

4. More teeth were affected in the maxilla than in the mandible.

We wish to express our deepest gratitude to Col. A. Azmon, Surgeon General of the Israel Defense Army, for his constant help and deep interest in dental affairs. We also wish to thank Drs. I. Schour and M. Massler of the University of Illinois College of Dentistry for their help and advice.

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THE FOOD HABITS AND DENTAL STATUS OF SOME CONNECTICUT CHILDREN

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THE DENTAL status and food habits of 864 children in eastern Connecticut were surveyed. Their rate of dental decay has been studied in relation to various factors in their diets, including consumption of fruits and vegetables, milk intake, consumption of candy and soft drinks, and between-meal eating. Information about their food habits was obtained from 7-day food intake records kept by the children.

In the 19 elementary schools in eastern Connecticut which volunteered to cooperate, all the children in grades 5 to 8 participated. Only those children with complete 7-day food intake records are included in this report. Of the 864 children with complete records, 368 were in attendance at 3 elementary schools in a city of 15,000 population. These have been designated as "city" children, in contrast to the other 496 children, who attended small village or rural schools and who have been designated as "rural" children.

The study was carried out in the spring of 1950. This was previous to any possible effect on these children's teeth of the fluoridation of public water supplies or of the topical application of fluorides.

The diets of the children, when rated as to intake of the basic 7 foods,[1] were found to be moderately good, on the average, with none up to standard every day and probably none seriously deficient. The greatest deficiency was in the intake of fruits and vegetables. More detail about the quality of their diets has been published elsewhere.[2]

DENTAL STATUS OF THE SUBJECTS

Dental examinations of these children were made by 2 public health dental hygienists of the Dental Hygiene Division of the State Department of Health. The examinations included the use of good light, mouth mirrors, and explorers, but no x-ray.

The average number of decayed, missing, and filled teeth per child (DMF rate) at various age levels, for city and rural children, and for boys and girls in the present study is shown in Fig. 1, *A* and *B*, in comparison with a curve for the dental status of 19,906 children in Connecticut in 1937-44.[3] There seems to be an increase in the rate of dental decay between 1944 and 1950, beginning at about 14 years of age.

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The rate of dental decay in the city children in the present study is higher than that of the rural children at all age levels (Fig. 1, *A*). The average DMF rate for city children is 9.1; for rural children, 7.1.

The difference between the rate of dental decay in boys and that in girls in the present study seems to fluctuate from year to year, with no consistent sex difference (Fig. 1, *B*). The average number of DMF teeth for the boys is 8.1; for the girls, 8.0.

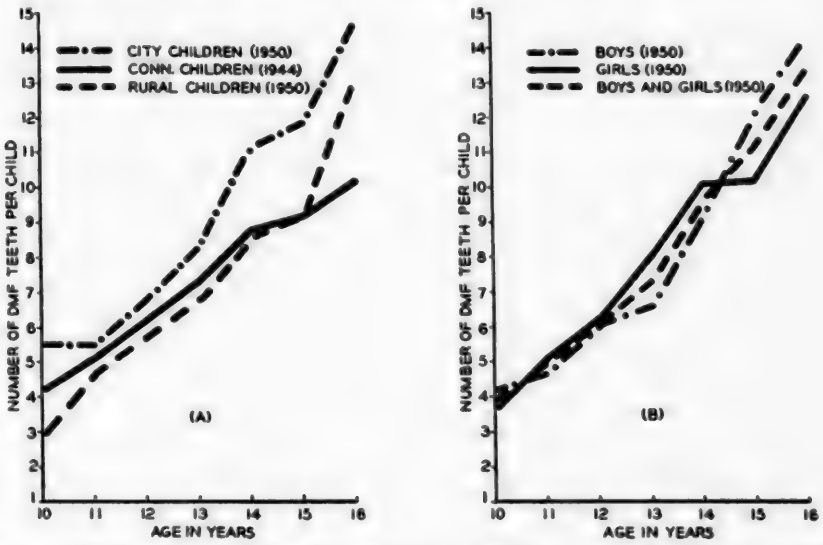

Fig. 1.—Average DMF rate of children at various age levels. *A*, For city and rural children in 1950, as compared with the rate for all children in 1944; *B*, for boys and for girls in 1950.

RELATIONSHIP BETWEEN DENTAL STATUS AND DIET

Scoring and Rating the Diets.—The diets of the 864 children were scored and rated as to content not only of the basic 7 food groups, but also on their content of many individual food items, including candy and soft drinks as well as frequency of between-meal snacks.

In scoring the diets for degree of nutritional adequacy on the basis of the National Food Guide,[1] the following numbers of servings of the various food groups were considered to constitute an adequate ("optimal") diet for a child:

Group	*Foods*	*Number Servings per Day*
1	Leafy, green, and yellow vegetables	1
2	Citrus fruit, tomatoes, raw cabbage, and other high vitamin C foods	1
3	Other vegetables and fruits, including potatoes	2
4	Milk, cheese, and ice cream (cups of milk, including milk equivalent of other dairy products)	4
5	Meat, poultry, fish, eggs, dried beans and peas, nuts	2
6	Bread, flour, and cereals, whole-grain or enriched or restored	2
7	Butter and fortified margarine	1
	Total number of servings	13

Additional servings of any food group were not accepted in lieu of shortages in other groups the same day or for lacks in the same group on other days. The number score for an adequate diet was 13.

The diets were divided into 3 groups as to nutritional adequacy: high, middle, and low. Children in the high diet rating group had an average daily diet scores ranging from 9.0 to 13 points, inclusive, per day. No child received an average score as high as 13 though some had scores of 13 on individual days. Diets in the middle diet rating group scored 7.0 to 8.9 points, inclusive; those in the low diet rating group, 3.0 to 6.9 points, inclusive. The purpose of the division at these points was to avoid too large or too small a number in each of the groups. The distribution of the city children and of the rural children among the 3 diet rating groups is indicated in Table I.

TABLE I

DISTRIBUTION OF CITY AND RURAL CHILDREN AMONG THE THREE BASIC SEVEN DIET RATING GROUPS

DIET RATING GROUPS	DIET SCORE (RANGE)*	CITY CHILDREN		RURAL CHILDREN		ALL CHILDREN	
		NUMBER	PER CENT	NUMBER	PER CENT	NUMBER	PER CENT
High	9.0-13	93	25	265	54	358	41
Middle	7.0-8.9	154	42	163	33	317	37
Low	3.0-69	121	33	68	13	189	22

*A score of 13 points per day indicates an adequate ("optimal") diet; it includes the recommended number of servings of all the basic 7 food groups.[1]

Dental Status in Relation to Basic Seven Diet Score.—The relative dental status of children in the high, middle, and low basic 7 diet rating groups is shown in Table II. It can easily be seen that among the children in the high diet rating group (score range: 9.0 to 13) there is a higher percentage in the best dental class (with 0 to 6 DMF teeth per child) and a lower percentage in the poorest dental class (with 13 to 24 DMF teeth per child), as compared with the distribution of the children in the low diet rating group (score range: 3.0 to 6.9). There is obviously a positive relationship in these children between present dental status and general "goodness" of the diet as determined from their 7-day food intake records.

TABLE II

DISTRIBUTION OF CHILDREN AMONG THE THREE DIET RATING GROUPS AND THE THREE DENTAL STATUS GROUPS

DIET RATING GROUPS	DIET SCORE (RANGE)	RANGE OF DMF RATES IN THE THREE DENTAL STATUS GROUPS					
		0-6		7-12		13-24	
		NUMBER	PER CENT	NUMBER	PER CENT	NUMBER	PER CENT
High	9.0-13	208	58	122	34	28	8
Middle	7.0-8.9	127	40	111	35	79	25
Low	3.0-69	62	33	76	40	51	27

Dental Status in Relation to Fruit and Vegetable Consumption.—It can be seen from Table III that those children eating 4 or more servings of fruits and vegetables per day had, on the average, a lower rate of dental decay than those eating only 0.1 to 1.9 servings per day.

Dental Status in Relation to Milk Consumption.—The dental status of these children in relation to 3 different levels of milk intake is reported in Table IV. Of the children drinking 4 or more cups of milk (including milk equivalent of other dairy products) per day, a higher percentage are in the best dental group and a lower percentage are in the poorest dental group, as compared with the children in the lowest milk intake group.

TABLE III

RELATIONSHIP BETWEEN DENTAL STATUS AND TOTAL FRUIT AND VEGETABLE CONSUMPTION

RANGE OF DMF RATES OF CHILDREN IN THE THREE DENTAL STATUS GROUPS:	PERCENTAGE DISTRIBUTION OF CHILDREN AMONG THE THREE DENTAL STATUS GROUPS		
	0-6 (%)	7-12 (%)	13-24 (%)
Total number of servings of fruits and vegetables per day:			
4 or more	54	34	12
2.0-3.9	44	44	12
0.1-1.9	30	40	30

TABLE IV

RELATIONSHIP BETWEEN DENTAL STATUS AND THREE LEVELS OF MILK CONSUMPTION

RANGE OF DMF RATES OF CHILDREN IN THE THREE DENTAL STATUS GROUPS:	PERCENTAGE DISTRIBUTION OF CHILDREN AMONG THE THREE DENTAL STATUS GROUPS		
	0-6 (%)	7-12 (%)	13-24 (%)
Number of cups of milk* per day:			
4 or more	51	37	12
2.0-3.9	46	40	14
0.1-1.9	35	37	28

*Including milk equivalent of other dairy products.

TABLE V

DENTAL DECAY RATE IN RELATION TO LEVEL OF MILK CONSUMPTION

AVERAGE NUMBER OF CUPS OF MILK PER DAY:*	DMF RATES OF CHILDREN IN VARIOUS MILK CONSUMPTION GROUPS											
	0.1-1.0		1.1-2.0		2.1-3.0		3.1-4.0		4.1-5.0		5.1-8	
	NUMBER OF SUBJECTS	DMF RATE	NUMBER OF SUBJECTS	DMF RATE	NUMBER OF SUBJECTS	DMF RATE	NUMBER OF SUBJECTS	DMF RATE	NUMBER OF SUBJECTS	DMF RATE	NUMBER OF SUBJECTS	DMF RATE
Age groups:												
11-12 years	12	7.33	43	6.76	94	5.78	122	5.73	86	4.77	44	5.25
13-14 years	16	12.31	41	9.25	95	8.30	109	8.12	74	7.37	44	8.89
9-16 years	31	10.65	93	8.16	211	7.09	254	7.03	177	5.95	96	7.12

*Including milk equivalent of other dairy products.

Table V shows how the average rate of dental decay varies with the specific number of cups of milk consumed per day. In Fig. 2 the DMF rate is plotted against the average number of cups of milk (or dairy equivalent) consumed per day. There is a marked improvement in dental status with increase in level of milk intake from 0.1 to 1.0 cup to 1.1 to 2.0 cups per day. A further drop in DMF rate is noted with an increase to 2.1 to 3.0 cups of milk per day. With an increase in daily milk intake to 4.1 to 5.0 cups there is again an improvement in

dental status in all 3 age groups. In contrast to this consistent improvement in dental status with increase in milk consumption from 1 cup or less to 4.1 to 5.0 cups, there is a definitely higher DMF rate in those children drinking more than 5 cups a day.

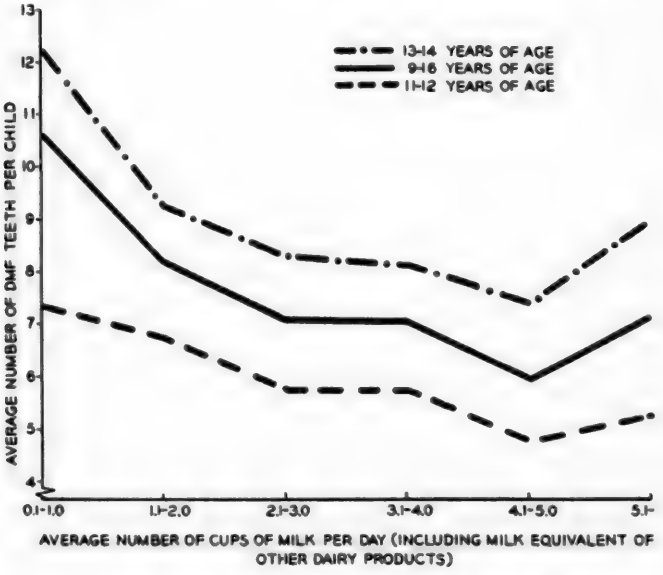

Fig. 2.—Dental decay rate in relation to level of milk consumption.

In looking for a possible reason for this higher dental caries rate in the children who drank over 5 cups of milk a day, as compared with those who drank 4 to 5 cups, several factors were considered. Their diets might have rated differently in other factors found here to be related to caries, namely, the intake of fruits and vegetables and of refined sweets, and the frequency of between-meal eating. However, on comparing the quality of the diets of the "heavy milk drinkers" with the diets of the group as a whole, it was noted that the former compared favorably with the latter in average daily intake of green and yellow vegetables, of high vitamin C fruits and vegetables, and of total fruits and vegetables.

The intake of candy and soft drinks and the frequency of between-meal eating in the heavy milk drinkers was compared with those who drank 4.1 to 5.0 cups of milk a day. The average daily intake of candy and soft drinks, combined, in this group was 0.94 times; in the group drinking 4.1 to 5.0 cups of milk per day it was 0.84. The average number of between-meal snacks per day in the 2 groups was 1.28 and 1.14, respectively. It is doubtful whether this slightly higher level of candy, soft drinks, and between-meal snacks in the high milk intake group could account for their higher rate of dental decay.

Another possible cause for the higher incidence of dental decay in this group of heavy milk drinkers might be a relatively greater representation of the

older age levels with a naturally higher DMF rate. This was found not to be the case to any marked degree. The average age of the heavy milk drinkers was 12.47 years, of those getting 4.1 to 5.0 glasses a day, 12.25 years. Neither did the group of heavy milk drinkers include a higher percentage of city children with the higher average DMF rate as compared with rural children. The percentage of city children in the 2 groups was 39.8 and 42.6, respectively. It was thought the higher average DMF rate in the high milk intake group might be due to an unusually high rate in a small percentage of the group. However, the distribution curve for the DMF rate in this group is similar to the distribution curve of the whole group.

A high milk intake by children with an already high rate of dental decay may be the result of advice from a dental hygienist, dentist, or school nurse. Another reason might be a higher incidence of certain childhood febrile diseases with a consequent increase in dental decay, followed by advice from a pediatrician to "drink lots of milk" with the intent of combating the assumed harmful effect of the disease on the teeth. In neither of these cases, of course, would the high milk intake be a causative factor in the production of the high DMF rate.

Dental Status in Relation to Other Dietary Factors.—The dental status of the children was studied in relation to extent of between-meal eating and to the total intake of candy and soft drinks. In both cases a slightly better dental status was found with the lower intake, although the influence of these dietary factors was less marked than was the case in the first 3 comparisons made here (with basic 7 rating, with fruit and vegetable consumption, and with level of milk intake). The completeness and reliability of the children's records in regard to between-meal snacks and to total consumption of sweets and bottled soft drinks may perhaps be questioned even more than their records of intake of other foods. Such items could easily be overlooked, or could be considered as not being "food," or could be left off the record by a sensitive child in order to avoid possible criticism.

SUMMARY

The dental status of 864 Connecticut children (in grades 5 to 8) was studied in relation to the adequacy of their diets, as determined from 7-day food intake records kept by the children.

The average number of DMF teeth was slightly higher in these children at ages 14 and 15 than in Connecticut children of the same age reported in 1944.

There was no difference between the DMF rate for boys and that for girls. The former was 8.1; the latter, 8.0.

The rural children in this group had a slightly lower DMF rate (an average of 7.1 per child) than the city children (an average of 9.1 per child).

The children with the better diets (rated according to the National Food Guide of the United States Department of Agriculture) had lower DMF rates, on the average. A higher intake of fruits and vegetables was also associated with a lower DMF rate.

There was a marked and consistent drop in DMF rate with increase in number of cups of milk consumed, from an average of 10.65 DMF teeth for those

drinking less than 1 cup of milk per day to 5.95 for those drinking 4.1 to 5.0 cups per day. Those drinking over 5 cups per day had a slightly higher average rate of dental decay (7.12 DMF teeth). Possible causes for this are discussed.

Only a slight positive relationship was noted, in these children, between DMF rate and frequency of between-meal snacks; likewise between DMF rate and total consumption of candy and soft drinks. The possible incompleteness of the children's records as to these food items is discussed in connection with the low level of relationship to dental status noted in these subjects.

Credit is due Mrs. Beatrice Cohen for assisting with the dental examinations, and to Marilyn Oberle and Marie Gabriel for help in calculating and tabulating the data. Credit and appreciation are due the children who participated in the study and the teachers who supervised the diet records.

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MOUTH HYGIENE HABITS AND THEIR RELATION TO PERIODONTAL DISEASE

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THERE are few data in the literature on the relationship between toothbrushes and dentifrices, and periodontal disease. The population of Bombay, with their varied habits of oral hygiene, was used as a group for a survey of these relationships.

The individuals studied used the finger, the datan (a fresh twig chewed at one end to develop a brush form), or the toothbrush with tooth powders, tooth pastes, tobacco, charcoal, salt, or rice husk ash. The brands of commercial dentifrices were not recorded. The incidence of calculus and of periodontal disease has been shown to be high in Bombay.[1, 2]

A total of 1,445 men and women between 16 and 50 years of age were examined. The individuals were in the same general economic group, earning 80 to 100 Rs. per month. They were all relatively healthy as judged by ability to carry on their normal day-to-day work. Individuals with missing teeth, other than third molars, were not included.

EXAMINATION AND RECORDING

The examination was carried out with the use of the exploring tine and mouth mirror in natural light. The patient was questioned regarding the method of oral hygiene, and the answer was recorded.

The occurrence of periodontal disease was recorded as follows: The mouth was arbitrarily divided into 6 segments, 3 in each jaw. The middle segment extended from one canine region to the other, with right and left posterior regions on either side. The gingiva and teeth were examined for the usual clinical signs of periodontal disease (color, swelling, bleeding, recession, pockets, tooth mobility, etc.). No radiographic studies were made.

The presence of local and general periodontal disease was recorded separately. In instances where more than two margins or papillae in a segment were involved, the disease was classified as generalized for that segment. If one or two were involved, the disease was considered as localized. For the purposes of this survey only the generalized lesions were considered.

DATA

Table I gives the results of the survey. Eighty-three per cent of the individuals examined had generalized periodontal disease. Over half the patients used the fingers and tobacco as oral hygiene aids. Of the 398 individuals over 30 years of age, 373 had generalized periodontal disease.

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TABLE I

PERCENTAGE INCIDENCE OF GENERALIZED PERIODONTAL DISEASE IN EACH ORAL HYGIENE TECHNIC GROUP

AGE GROUPS	PERCENTAGE OF GENERALIZED PERIODONTAL DISEASE IN EACH AGE GROUP	PERCENTAGE INCIDENCE OF GENERALIZED PERIODONTAL DISEASE					
		TOOTHBRUSH-PASTE USERS	TOOTHBRUSH-POWDER USERS	FINGER-CHARCOAL USERS	FINGER-POWDER USERS	FINGER-TOBACCO USERS	DATAN USERS
16-20	52.0 (96)*	61.5 (26)*	45.5 (11)*	57.1 (7)*	57.1 (14)*	60.0 (5)*	42.4 (33)*
21-25	76.2 (476)	55.8 (77)	57.1 (21)	69.0 (58)	61.1 (36)	88.1 (253)	71.0 (31)
26-30	87.6 (485)	55.0 (40)	87.5 (16)	88.4 (26)	81.5 (27)	94.1 (326)	74.0 (50)
31-35	94.5 (238)	81.3 (16)	77.8 (9)	100.0 (7)	94.7 (19)	98.0 (199)	89.4 (38)
36-40	90.7 (118)	100.0 (15)	100.0 (5)	92.9 (14)	93.7 (16)	93.5 (31)	81.0 (37)
41-45	97.3 (38)	83.3 (6)	100.0 (2)	100.0 (7)	100.0 (9)	100.0 (3)	100.0 (11)
46-50	100.0 (4)	100.0 (3)	100.0 (1)	– (–)	– (–)	– (–)	– (–)
Total	83.2 (1455)	(183)	(65)	(119)	(121)	(767)	(200)

*The numbers in parentheses indicate the total numbers of individuals in each group, with or without generalized periodontal disease.

Statistical analysis by the "Analysis of the Variance Test" showed no significant difference, even at the 1 per cent level, in the incidence of generalized periodontal disease in toothbrush-powder users and toothbrush-paste users. However, there were significant differences in the occurrence of periodontal disease between the different age groups of toothbrush users. Among the finger users, there was no significant difference whether charcoal, powder, or tobacco was used but, again, the age group differences within the oral hygiene agent groups were significant.

The toothbrush users (with paste or powder), the finger users (with charcoal or powder) and the datan users were compared as groups. The finger-tobacco users were omitted to obtain groups of comparable size. There were no significant differences in the occurrence of generalized periodontal disease among these groups, although the age group differences were significant.

Using the Chi Square test the incidence of periodontal disease for finger-charcoal, finger-powder and finger-tobacco users was analyzed by segments. In the anterior segments there were no significant differences but for all 4 posterior segments the greatest occurrence of periodontal disease was among the finger-tobacco users. A similar comparison, by segments, of the effect of the finger, toothbrush, and datan as oral hygiene aids showed no significant effects.

SUMMARY

An incidence of 83.2 per cent of "generalized periodontal disease" was found among 1,455 residents of Bombay. The incidence increased with age. Most of the individuals examined used tobacco on the finger as an oral hygiene aid. Among the 248 toothbrush users no difference in effect was evident between tooth paste and tooth powder employment. Among the 1,007 finger users the use of charcoal, tooth powder, or tobacco as aids did not seem to effect the incidence of disease. However, the occurrence of periodontal disease was more common in the posterior segments of the mouths of finger-tobacco users than in those using the finger with tooth powder or charcoal.

The incidence of generalized periodontal disease did not appear to be affected by the method of oral hygiene but increased with age regardless of the oral hygiene method used.

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URINARY ESTROGEN IN PERIODONTOSIS

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SINCE periodontosis occurs predominately in females of reproductive age, it was thought that a study of the estrogen excretion might throw light on the etiology of this disease. In a previous study[1] of systemic factors in periodontosis, we found that the urinary neutral 17-ketosteroids, an index of androgen production, were within the normal range in 33 female patients. The determination of the 17-ketosteroids in urine is essentially a measure of adrenocortical and male gonadal activity. It has been shown that these glands are related to the activity of the osteoblasts. The mean value of the periodontosis group (33 cases) was 9.8 mg. per 24 hours and that of a control group (20 cases), free from periodontal disease, was 11.3 mg. per 24 hours. The difference between the means divided by the standard error of the difference was 1.44, indicating that there is no significant difference between the groups. However, 30 per cent of the cases in the control group gave values below 9.3 mg. per 24 hours, as compared with 52 per cent in the periodontosis group.

The present study is concerned with the urinary estrogen in 18 female subjects, 21 to 37 years of age in various stages of periodontosis. Urinary estrogen was determined by the fluorometric method of Jailer,[2] which is based on the finding of Marrian,[3] that when concentrated sulfuric acid is added to estrone, an orange color with greenish fluorescence is produced. Twenty-four-hour specimens were collected generally on the seventh day of the menstrual cycle (this will be referred to as the first specimen), and between the fourteenth and eighteenth day of the cycle, that is, on the presumed day of ovulation as assessed by the patient from her temperature record (this will be referred to as the second specimen). The usual range of urinary estrogen excretion in the absence of pregnancy is considered to be from 15 to 50 mcg. per 24 hours.

The degree of periodontosis was graded as follows: slight—showing loss of bone in specific areas but no need of immediate extraction of teeth; moderate—immediate extraction indicated of some teeth, which can be replaced by partial dentures; severe—extraction of all but six teeth indicated. Three cases were graded as slight, 8 as moderate, and 7 as severe.

In the 7 cases graded as severe, the estrogen values in the second specimen ranged from 14.7 to 28.3 mcg. per 24 hours, and were all lower than those in the groups graded as slight or moderate. In these latter groups the range of values in the second specimens was from 33.8 to 50.5 mcg. per 24 hours.

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RESULTS

Estrogen excretion in mcg. per 24 hours is given in Table I.

TABLE I

URINARY ESTROGEN IN PERIODONTOSIS

AGE	DEGREE OF PERIODONTOSIS	DAY OF CYCLE ON WHICH URINE WAS COLLECTED	ESTROGEN EXCRETION IN MICROGRAMS PER 24 HOURS
30	Slight	7	40.1
		18	38.2
34	Slight	8	28.3
		14	50.5
31	Slight	7	21.2
		14	50.4
37	Moderate	7	35.7
		14	33.8
31	Moderate	7	40.8
		14	44.6
29	Moderate	7	34.5
		14	34.8
30	Moderate	7	20.4
		16	42.1
25	Moderate	11	33.9
		14	41.9
37	Moderate	7	28.4
		18	48.5
21	Moderate	7	26.2
		15	47.2
22	Moderate	7	41.6
		15	48.7
34	Severe	7	26.0
		15	25.2
27	Severe	7	21.2
		15	23.3
34	Severe	7	21.0
		15	25.9
34	Severe	7	23.4
		16	28.3
31	Severe	7	32.0
		15	28.2
36	Severe	7	14.5
		15	21.2
25	Severe	9	11.6
		15	14.7

Lower values were found, although not consistently, in the first specimens also, in the severe periodontosis group. In these first specimens the range was from 11.6 to 32.0 mcg. per 24 hours for the severe periodontosis group, and 20.4 and 41.6 mcg. per 24 hours for the combined slight and moderate periodontosis groups.

SUMMARY

Urinary estrogen values in patients with periodontosis were in general in the normal range. However, the values were usually lower in the severe cases

than in the slight or moderate cases. This was consistently so in the severe cases in the specimens of urine collected on the presumed day of ovulation as assessed by the patient from her temperature record.

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THE NATURE OF THE GINGIVAL PLAQUE IN THE HAMSTER—PRODUCTION, PREVENTION, AND REMOVAL

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WHEN the hamster is fed a finely divided, high carbohydrate diet for approximately 100 days, measurable amounts of periodontal tissue destruction are the result.[1] This condition is due to supra- and subgingival accumulations which also may be intimately associated with cervical caries of the molar teeth.[2] These accumulations have been referred to as "plaque,"[3, 4] "tarter,"[5] and "food debris."[2] In our experience, this material has never appeared to be calcified.

It was noted that the subgingival accumulations were not apparent grossly in animals fed a coarse laboratory chow, nor was it formed when the chow was ground fine enough to pass through a 20-mesh screen.[6] In connection with caries studies, Orland[7] has found 5 varieties of lactobacilli and other aciduric microorganisms in the hamster. Lactobacilli were demonstrated and counted by Helman and Mitchell.[8] These facts seemed to indicate that the subgingival material might actually consist of masses of aciduric bacteria, among others. This study was conducted in order to discover the true nature of this material hereinafter referred to as "plaque."

EXPERIMENTAL PROCEDURES AND RESULTS

Eleven animals were maintained on a high carbohydrate diet* for 3 months. They were anesthetized and examined under a dissecting microscope; the presence of marked amounts of gingival plaque material was recorded, in vivo (Figs. 1 and 2). The animals were then fed laboratory chow† for 10 days and reexamined. The plaque material had disappeared (Fig. 3). The animals were then placed on the high carbohydrate diet for 2 weeks and the plaque reappeared (plaque-positive). Chow was fed again for 2 weeks and the plaque disappeared again (plaque-negative). At various times during this experimental period the following tests were performed.

1. *Microscopic examinations* were made of smears made from the subgingival material of plaque-positive animals, and stained by Gram's method. Gram-positive rods resembling lactobacilli were common. Streptococci, staphylococci, and various pleomorphic organisms also were seen. These findings agreed with

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*Whole wheat flour, 30%; whole powdered milk, 30%; cornstarch, 20%; sucrose, 15%; alfalfa, 4%; NaCl, 1%.

†Purina Fox Chow.

Fig. 1.

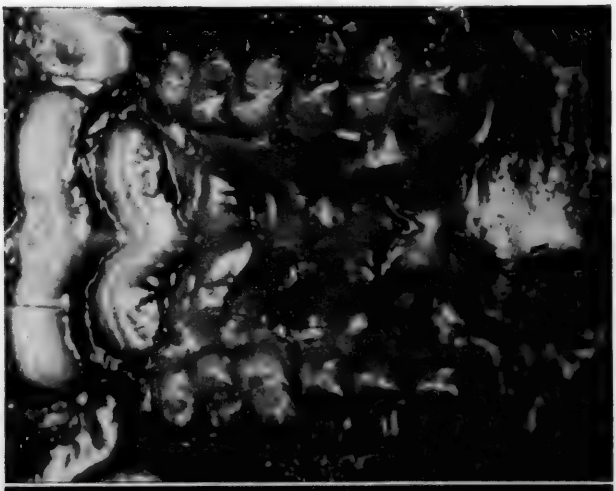

Fig. 2.

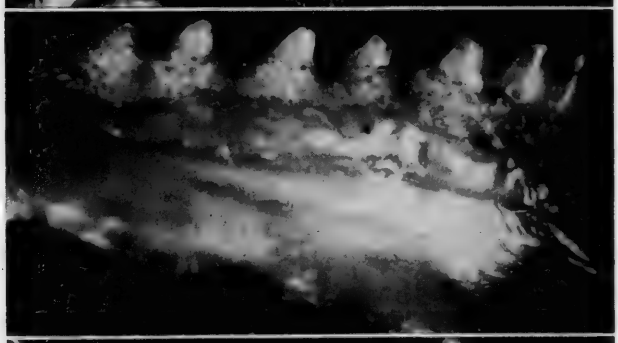

Fig. 3.

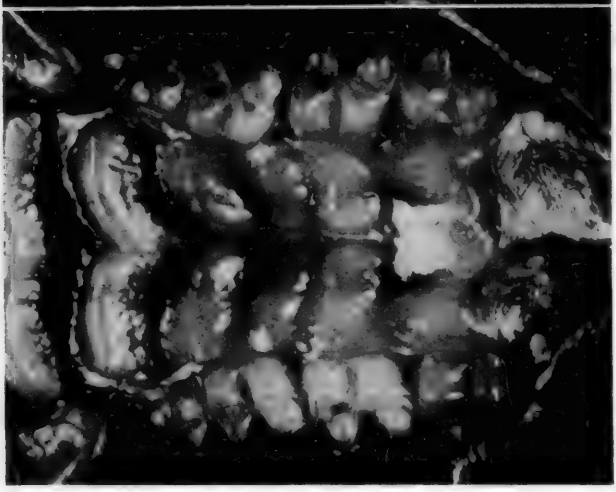

Fig. 1.—This photograph of the maxilla illustrates the findings in a "plaque positive" animal. Note the gray-white plaque on the palato-gingival surface of each molar—thus giving an in vivo, gross observational score of 6.

Fig. 2.—Plaque on the gingivae of mandibular molars. Difficult to observe in the living animal, and not "scored" in this study.

Fig. 3.—The maxilla of an animal which had previously been "plaque-positive" and is now negative after 2 weeks of a noncariogenic diet.

those of Orland.[7] We were impressed by the lack of debris in the smears—the body of the material seemed to consist of gross masses of microorganisms. Gingival smears from plaque-negative animals revealed similar microorganisms, but they were much less abundant.

2. *Plaque pH change* was observed.[9] The plaque material was taken directly from the plaque-positive hamster and placed on a glass slide. The material was covered with a few drops of aqueous methyl red .02% (pH range, 4.2 red to 6.3 yellow), and a few crystals of sucrose were added. In a matter of 15 to 30 seconds small islands of brilliant red appeared in the yellow solution, denoting acid production through bacterial enzymatic action on the sucrose, thus indicating the presence of acid-producing microorganisms.

3. *Glucose broth** at pH 7 was inoculated with material from plaque-positive animals, and after 2 days the pH had dropped to an average figure of 4.3 as compared to noninoculated control tubes with a pH of 6.8. This action was repeated with small amounts of material obtainable from plaque-negative animals with similar results, thus indicating the omnipresence of aciduric organisms in this area of the mouth.

4. *SL-agar plates*[10] were plated with dilutions (1:10, 1:100, 1:1000) of the glucose broth which had shown pH changes, and colony growth was seen in 24 hours. These colonies resembled those of lactobacilli and smears made from them showed gram-positive rods. The media used is highly selective for lactobacilli.

5. *Microscopic sections* of the plaque *in situ* on the molar teeth, stained with a modified Gram method[11] revealed the presence of masses of gram-positive rods and other organisms.

Penicillin Test.—In view of these findings it seemed reasonable that if plaque-positive animals were fed some agent that would affect the organisms involved, preferably a known caries preventive agent,[12, 13] the plaque should disappear in a short time. Therefore, 32 plaque-positive animals which had been maintained on a high carbohydrate diet for many weeks were selected and divided into a control and experimental group. Each animal had an in vivo gross observational "score" of 3 or more palatal gingival surfaces involved (6 possible, 1 area opposite each upper molar). These and subsequent scores were obtained without using anesthesia, in a carefully controlled, objective manner. Both groups were fed the high carbohydrate diet. The experimental group received, in addition, 500 units of sodium penicillin G/Gm. of diet, and 100 units/c.c. in the drinking water. Food and water were furnished ad libitum for 2 weeks. At the end of this period the palatal periodontal plaque scores in the living animal were as follows: controls, 3.98 average number of palatal, gingival surfaces per animal; experimental animals, .28 (total possible, 6). There was no appreciable sex difference, although the male scores ran a little higher than those of the females.

DISCUSSION

The plaque-positive animals ultimately suffer marked recession of periodontal tissues. The ease with which this plaque may be removed within 2 weeks,

*Yeast extract, ½%; tryptone, ½%; glucose, 1%; sodium thioglycollate, .05%; distilled H_2O to 100%; pH adjusted to 7.00 and autoclaved.

and re-induced in a like amount of time by diet control seems remarkable. The association of this form of periodontal disease with cervical "caries" in the hamster[2] raises the question of a possible relationship between such a subgingival plaque and cervical caries in human beings.

Waerhaug[14] has shown, in the human being, that subgingival "plaque" always contains bacteria, and he has been unable to demonstrate other materials such as food debris in this plaque. Similarly, we were unable to demonstrate any debris or material other than bacteria in the plaque of hamsters maintained on a high carbohydrate diet. Zander[15] studied calculus attachment to teeth and showed microorganisms at this site penetrating cementum and dentin. Could this have occurred after decalcification of these surfaces by aciduric organisms? The organisms demonstrated in situ were gram-positive rods and filamentous types of organisms.

Riffle[16] has demonstrated that softening of root surfaces occurs in cases of long-standing periodontal disease, and he expressed the belief that this softened portion of the root should be removed in order to cure the disease.

We believe that in the human being some forms of chronic periodontal disease and cervical root "caries," or "surface softening," or "deterioration"[16] sometimes may be associated with the same factor—a plaque consisting in part of aciduric microorganisms. Further studies are needed to define the possible role of proteolytic organisms in this region.

The plaque-test of penicillin described may lead to the development of a screening test for potential caries inhibiting agents. If hamsters were maintained on a high carbohydrate diet until they were plaque-positive, and then tested for 2 weeks with the addition of a possible caries inhibiting agent, a 2 week test may give additional, early, encouraging or discouraging information about the agent. Although further study of a variety of agents of an antibacterial, anti-enzyme, or other nature, known to be anti-cariogenic, will be required, it is believed that some degree of success will be forthcoming on the basis of this penicillin test, and previous reports from this laboratory which showed that periodontal plaque scores were reduced by feeding dibasic ammonium phosphate, or dibasic sodium phosphate, but not urea.[17] These results paralleled the caries scores in the same animals.[18] Related work recently reported by Costich[19, 20] indicated that copper, NaF, $NaFPO_3$, and a chlorophyll compound reduced alveolar crest resorption when fed to hamsters. Such resorption should be directly related to the accumulation of periodontal plaque.

SUMMARY AND CONCLUSIONS

Hamsters maintained on a high carbohydrate diet for some weeks develop a grossly apparent white plaque at the palato-gingival area of the upper molars which may be observed in vivo. When these animals were fed laboratory chow (noncariogenic) for 2 weeks, the plaque disappeared. After 2 subsequent weeks of a high carbohydrate diet the plaque reappeared. By feeding small amounts of penicillin with the high carbohydrate diet, the plaque was made to disappear. When the grossly detectable plaque was studied by direct microscopic examination, by pH change in vitro, by selective culture and other methods, it was

determined that the plaque consists, in large part, of aciduric microorganisms. The ease of production and "erasure" of the plaque may lead to a short-term screening test for possible caries inhibiting agents. Speculation is made on the possible application of these findings to dental caries and periodontal disease in the human being.

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AN IN VITRO STUDY OF ANTISEPTICS AND ANTIBIOTICS USED IN ENDODONTICS

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THE object of this study is to ascertain the action, in vitro, of some antiseptics and antibiotics which are used for the destruction of microorganisms in infected root canals.

The laboratory procedures are divided into three parts: (1) preliminary tests to determine the efficacy of the agents in general and to establish whether they are adapted to clinical practice; (2) a qualitative study by the disc method; and (3) a quantitative study by the serial dilution method.

In our laboratory, the flora of several infected root canals were found to be of a mixed type when several teeth were compared, but in most cases the root canals contained only one type of organism. The alpha streptococcus was found most frequently. In some cases 2 types of organisms were found, but 1 type exceeded the other in numbers. Mixed flora were nearly always observed in exposed root canals, or in the case of contamination during the course of endodontic treatment. A number of pulpless teeth with periapical areas were found to be sterile.

With the advent of antibiotics, it has become necessary to study the bacterial flora, as most antibiotics are selective in their action.

In our experiments, 4 types of microorganisms, *Str. fecalis, Staph. albus, C. albicans,* and *E. coli,* were used because they are most commonly found in infected root canals.

After some preliminary tests, Antistine, Asterol, sodium caprylate, decyl ammonium sulfate, fradicin, neomycin, Perazil, rimocidin, Terramycin, and thiolutin were selected for the tests. Of these, Antistine, Asterol, sodium caprylate, decyl ammonium sulfate, and Perazil are not antibiotics.

Jawetz, Gunnison, and Speck,[1] who studied the antagonism caused by Aureomycin, Terramycin, or chloramphenicol on the one hand, with penicillin on the other, showed that it occurs only if any of the former is given either with or before the latter, but not if penicillin is administered first. This seems to show that it is not a mutual antagonism or incompatibility of the drugs, but rather an interference by chloramphenicol, Terramycin, or Aureomycin with the activity of penicillin. Interference of effect is observed not only among antibiotics but also when an antibiotic is used with some other agent, such as penicillin with boric acid, or with sulfadiazine under certain conditions.

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On the other hand, several studies have demonstrated a synergistic effect among certain antibiotics such as between penicillin and streptomycin, and penicillin and bacitracin, and also between penicillin and some sulfonamides.

MATERIALS

Agents.—Antistine is a synthetic antihistaminic (2 phenyl-benzylamino-methyl-imidazoline hydrochloride) with relatively low toxicity. *Asterol* is the dihydrochloride of 2 methylamine-6-benzothiazole considered to have a high degree of activity against fungi, including *C. albicans*. *Sodium caprylate*, the sodium salt of caprylic acid, is relatively nontoxic. *Fradicin* is an antibiotic derived from *Streptomyces fradiae*. *Neomycin* is an antibiotic having a cyclo-ethanol type structure. *Perazil* is an antihistaminic which has a piperazine nucleus as a base instead of the ethylene group. *Rimocidin* is an antibiotic derived from *Streptomyces rimosus*. *Terramycin* is an antibiotic derived from *Streptomyces rimosus*. *Thiolutin* is an antibiotic derived from *Streptomyces albus*.

METHOD

Method A.—Cultures were prepared from a 24-hour growth of organisms grown in brain heart infusion broth for *Str. fecalis*, *S. albus*, and *E. coli*, and in Sabouraud's medium for *C. albicans*. The agents to be tested for their antibacterial effect were prepared in dilutions of 5 and 50 mg. per cubic centimeter of solvent. In addition, dilutions of 100 mg. per cubic centimeter of solvent were made in those cases where the chemical agents were to be used against *Str. fecalis* or *C. albicans* because of the greater resistance of these microorganisms. In those cases where the antibiotic or chemical agent was not soluble in water, it was first dissolved in a little propylene glycol and then in water. The amount of propylene glycol used was negligible from an antibacterial standpoint as determined by test. Graduated pipettes were used for transferring solutions of the chemicals or dilutions of the microorganisms. To each test tube was transferred 0.1 c.c. of the chemical solution and 0.1 c.c. of the suspension of microorganisms. Fresh pipettes were used for each transfer.

As controls, 40 tubes of culture medium were used containing the same amount of culture medium and the same amount of bacterial suspension, but without the addition of chemicals. The tubes were incubated at 37° C. except the Sabouraud's medium, which was incubated at room temperature. In those cases where there was no evidence of growth, subcultures were made.

Results: All cultures with *Str. fecalis* were positive when the chemicals were used in a concentration of 5 mg. per cubic centimeter. In a 50 mg. per cubic centimeter concentration, Antistine, decyl ammonium sulfate, and Terramycin cultures were negative while all others were positive. All cultures were positive against *C. albicans* except thiolutin in a concentration of 100 mg. per cubic centimeter. Neomycin, Perazil, Terramycin, and thiolutin in both 5 mg. and 50 mg. concentrations were effective against *Staph. albus*. All the rest showed growth. Against *E. coli*, Antistine, decyl ammonium sulfate, neomycin, Perazil,

Terramycin, and thiolutin showed no growth in both 5 mg. and 50 mg. per cubic centimeter concentration. Cultures with Asterol, sodium caprylate, fradicin, and rimocidin were positive on examination.

Method B.—In order to study the action of the chemical objectively, agar pour-plates were made containing 10 per cent of sterile horse blood, and the agar was then inoculated with a suspension of organisms against which the chemical solution was to be tested. Discs of sterile filter paper which had been submerged in a solution of the chemical for 24 hours previously and then dried thoroughly were placed on the surface of the agar immediately after its inoculation with the microorganisms. The plates were then incubated for 48 hours at 37° C., except Sabouraud's medium, which was kept at room temperature. Zones of inhibition were apparent in most cases after 24 hours and observations were made both after 24 and 48 hours. In addition to the chemicals previously tested, aerosporin, endomycin, humulon, lupulon, hexahydrolupulon, aspergillic acid, and gliotoxin were added to the tests.

Since one cannot determine microscopically whether a zone of inhibition is total or only partial, the zones were divided into small areas, working from the border of the disc to the periphery of the zone of inhibition. Samples were taken from each of these areas, transferred to culture medium, and incubated. Smears were then made for microscopic examination.

Results: It was found that in addition to partial or total inhibition, some chemicals produced two well-defined zones of inhibition, e.g., neomycin against *Str. fecalis,* gliotoxin against *E. coli,* aerosporin against *E. coli,* Perazil against staphylococcus, etc. In some cases a third zone appeared which may be considered a zone of stimulation. Sometimes this zone was situated between the zones of inhibition, at other times it was outside the zones of inhibition.

Method C.—After determining the qualitative effect of the chemicals, an effort was made to determine their quantitative effect. Only those chemical agents which appeared to be relatively effective were included in these tests.

The tests were carried out by the serial dilution method in the following manner: Seven tubes were each filled with 4.5 c.c. of brain heart infusion broth; 0.5 c.c. of a 24-hour culture of the selected organisms was added to the first tube, which was shaken to distribute the organisms evenly; 0.5 c.c. was then removed from the first tube and transferred to the second tube, and this procedure was continued until each of the tubes was inoculated. Then 0.5 of this inoculated medium was transferred to a tube containing molten agar, and a Petri plate was poured. This was repeated in triplicate for each tube for a total of 21 plates. Incubation was carried out at 20° C., 37° C., and 45° C. for 48 hours. It was found that 37° C. was the optimum temperature for growth, followed by 20° C. and 45° C., respectively. Little difference was found between brain heart infusion broth and Sabouraud's medium for growing *C. albicans.* The number of colonies which developed upon these plates was counted over a Frosh counter. Where the colonies were numerous, only one-fifth of each dish was counted and multiplied by 5. An average of the 3 plates was taken in each case. In some cases, counting was impossible owing to the large number of colonies.

SUMMARY AND CONCLUSIONS

1. A number of antibiotics and other chemical agents were tested against 4 types of microorganisms commonly found in infected root canals, *Str. fecalis, Staph. albus, E. coli,* and *C. albicans.*

2. Fradicin, thiolutin, sodium caprylate, and Asterol were effective against *C. albicans,* the most effective being fradicin.

3. Of those agents tested, decyl ammonium sulfate and neomycin were most effective against *E. coli.*

4. Terramycin and Perazil were effective against both *Staph. albus* and *Str. fecalis.*

5. The possibility of combining 2 or more agents in order to produce a wider antibacterial spectrum than each has commends itself. Such factors as compatibility, stability, possible irritant effect, etc., would need to be taken into consideration.

6. The more commonly used antibiotics, such as penicillin, streptomycin, or bacitracin, were not included in these tests as their antimicrobial effect has been studied by others.

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